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THE INFLUENCE OF STEROIDS ON HOST DEFENSE AGAINST *Trypanosoma danilewskyi* (LAVERAN & MESNIL, 1904) IN THE GOLDFISH *Carassius auratus* (L.)

BY

RIJIAN WANG ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science.

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1994.

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "THE INFLUENCE OF STEROIDS ON HOST DEFENSE AGAINST *Trypanosoma danilewskyi* (LAVERAN & MESNIL, 1904) IN THE GOLDFISH *Carassius auratus* (L.)" submitted by Rijian Wang in partial fulfillment for the degree of Master of Science.

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ABSTRACT

An understanding of the mechanisms of host defense against pathogens is essential for prevention and control of certain fish diseases. It is known that fish are more susceptible to infectious diseases and have higher mortality when they are under stress. During spawning fish are under stress, which is characterized by drastic increase of steroids in the blood. The purpose of this thesis was to test the hypothesis that sex steroids affect the susceptibility of goldfish to *Trypanosoma danilewskyi* infection.

In vitro assays were developed to test the effects of steroids on the antimicrobial activity of goldfish lymphocytes and macrophages. Goldfish lymphocytes proliferated in response to stimulation by phorbol 12-myristate acetate and calcium ionophore. The cultured goldfish macrophages functionally resembled mammalian macrophages determined after assessment of chemotaxis, phagocytosis, and production of reactive oxygen and nitrogen intermediates.

The course of *T. danilewskyi*-infection in goldfish and mortality induced by the parasites were examined. Goldfish responded to *T. danilewskyi* infection in a dose-dependent manner: larger inocula of the trypanosomes caused higher mortality.

Estradiol and cortisol suppressed mitogen-induced proliferation of goldfish lymphocytes and inhibited chemotaxis and phagocytosis of goldfish macrophages. Cortisol, but not estradiol inhibited nitric oxide production of goldfish macrophages. Neither steroids influenced O_2^- production of goldfish macrophages.

The *in vivo* effects of estradiol on goldfish defense against *T. danilewskyi*-infection were determined. Estradiol significantly increased the parasitaemia and mortality in primary but not in challenge infection of goldfish with *T. danilewskyi*. Estradiol administration suppressed the mitogen-induced proliferation of goldfish lymphocytes.

The findings of this study demonstrate that estradiol and cortisol suppress immune responses of goldfish *in vitro* and *in vivo*. These data may partially explain why fish are more susceptible to infectious diseases during spawning.

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LIST OF ABBREVIATIONS

A23187	calcium ionophore
B cell	thymus-independent lymphocyte
C_{1-9}	component 1-9 of complement
Con A	concanavalin A
CPM	count per minute
CRP	C-reactive protein
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EAGFS	endotoxin activated goldfish serum
Fc	Fc fragment of immunoglobulin
GFLM	goldfish leukocyte medium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IFN γ	interferon γ
IgM	immunoglobulin M
IL-1	interleukin-1
LM	light microscope
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
MOF	microscopic oil fields
MS222	tricaine methanesulfonate
MW	molecular weight
NADPH	nicotinamide-adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NCC	nonspecific cytotoxic cells
NK	natural killer cells
NOS	nitric oxide synthase
O. D.	optical density
O_2^-	superoxide anion
PBL	peripheral blood leukocytes
PHA	phytohemagglutinin
PMA	phorbol 12-myristate acetate
PRCV	packed red cell volume
RBC	red blood cells

S. E. M.	standard error of the mean
SDS	sodium dodecyl sulfate
sIg^+	surface immunoglobulin positive
sIg^-	surface immunoglobulin negative
SRBC	sheep red blood cells
T cell	thymus-dependent lymphocyte
TEM	transmission electron microscope
TDM	*Trypanosome danilewskyi* culture medium
$[^3H]$	tritium-labelled

Chapter One: General Introduction

A. INTRODUCTION

Infectious diseases are major contributors to the losses in fish production, particularly in fresh water fish farming. Intensive fish aquaculture practices induce stress in fish, resulting in higher susceptibility of fish to infection [Woo and Jones, 1987]. A better understanding of the mechanisms of host defense against pathogens is essential for the prevention and control of certain fish diseases. The successful control of infectious diseases in fish should reduce mortality and increase productivity of fish farming.

It is well known that fishes, like mammals and birds, are more susceptible to infectious diseases when they are under stress, which usually results in higher mortality [Ellis, 1981; Grimm, 1985]. During spawning, fish are under stress due to contributing factors such as improper food intake, severe physical exercise, and damage to the skin and body [Pickering, 1981]. The most important response in spawning-induced stress is a drastic change in the levels of hormones in fish blood: both male and female sex hormones increase significantly shortly before and during spawning [Kobayashi *et al.*, 1987; 1988].

The immunosuppressive effects of adrenal steroids on the immune system have been reported in plaice, *Pleuronectes platessa* (L.) and brown trout, *Salmo trutta* (L.) [Grimm, 1985; Pickering and Duston, 1983]. No data are available concerning the immunosuppressive effects of adrenal steroids on the immune responses of goldfish. The immunosuppressive effects of sex steroids on mammalian immune responses are well documented [Grossman, 1984; 1985; Magnusson and Fossum, 1992]. However, little is known about the effects of sex steroids on fish immune responses against parasites.

Goldfish were used in this research because: (1) goldfish are closely related to carp, the economically important fish species used in aquaculture in Asia; (2) they are susceptible to *Trypanosoma danilewskyi*; and (3) goldfish are easy to maintain and handle, and their natural mortality is relatively low under the laboratory conditions.

To examine the sex steroid- and adrenal steroid-immune system interaction in goldfish host defense against pathogens, I used a *T. danilewskyi*-goldfish host-parasite model. *T. danilewskyi* is a fish haemoflagellate that naturally infects common carp, *Cyprinus carpio* (L.), and related species in Eastern Europe [Woo,

1981a]. Successful experimental infections of goldfish with *T. danilewskyi* have been reported by Lom [1973a; 1973b] and Woo [1981b]. This parasite induces a dose-dependent mortality in experimentally infected goldfish [Woo, 1981b].

B. THE THESIS

The research described in this thesis was conducted to examine the effects of sex steroid estradiol and adrenal steroid cortisol on immune responses of goldfish against *T. danilewskyi*.

Chapter two is a brief review of literature relevant to my research project and chapter three describes the development of the immunological assays for goldfish lymphocytes and macrophages used in most of the experiments. In chapter four I describe the characteristics of *T. danilewskyi* infection in goldfish. The *in vitro* effects of estradiol and cortisol on the functions of goldfish lymphocytes and macrophages are described in chapter 5, and the *in vivo* effects of the steroids on the course of *T. danilewskyi* infection and on mitogen-induced lymphocyte proliferation are examined in chapter 6. The last chapter is a synopsis and general discussion of the findings.

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Chapter Two: Literature Review

A. TELEOST IMMUNE SYSTEM

The immune system plays a critical role in host defense against infectious diseases and in maintaining homeostasis in fish. Like other vertebrates, fish possess both non-specific (innate) and specific (acquired) immune responses. The innate or non-specific immune responses operate in the absence of specific immune responses and are responsible for the resistance to certain pathogens. The acquired or specific immune responses develop after contact with the pathogen, and regulate the elimination of the pathogens from the host and resistance to the secondary infection.

The effectors of innate humoral immune responses include a number of protein molecules such as complement [Jensen *et al.*, 1981], interferons, chitinase, precipitins, lysins, agglutinins, lysozyme and the C-reactive protein-like substance in the blood or mucus on the body surface [Ingram, 1980; Litman *et al.*, 1970; Murray and Fletcher, 1976; Winkelhake and Chang, 1982]. The effectors of non-specific cell-mediated immune responses include nonspecific cytotoxic cells (NCC), a functional analogue of natural killer (NK) cells in mammals and birds, macrophages, granulocytes and thrombocytes.

Cells of the Immune System of Fish

The first well-defined cell stage in differentiation of fish immune cells is the haemoblast, which is found in hematopoietic tissues such as the head-kidney and spleen. All the blood cells such as lymphocytes, macrophages and granulocytes are derived from the hemoblasts [Ellis, 1977].

Macrophages: Three types of macrophages are present in fish: 1) free rounded cells, resembling monocytes, in circulation; 2) reticulo-endothelial cells lining blood sinuses; and 3) melano-macrophages in the peritoneal cavity, liver, spleen and kidney [Agius, 1985]. Fish macrophages behave like mammalian macrophages: they adhere to glass and plastic, are actively motile, and phagocytic. They do not possess specific antigen receptors but can bind, ingest and degrade virtually any type of antigen [Graham *et al.*, 1988; Secombes *et al.*, 1988]. Macrophages in the spleen, kidney, endocardium, peritoneum and gill epithelium play a major role in elimination of particulate antigens [Goldes *et al.*, 1986]. Like

mammalian macrophages, specific phagocytosis of bacteria by fish macrophages is enhanced by opsonization with complement or specific antibody. The enhancement of phagocytosis of opsonized targets suggests that fish macrophages may have complement and Fc receptors [Griffin, 1983; Sakai, 1984]. Fish macrophages produce and release monokines such as interleukin-1-like (IL-1-like) molecule [Graham and Secombes, 1988] and acute phase molecules such as C-reactive protein [Kodama *et al.*, 1989]. The effector molecules for cytotoxicity of fish macrophages are both reactive oxygen and nitrogen intermediates [Chung and Secombes, 1988; see Chapter 3].

Lymphocytes: Fish lymphocytes are present in blood, spleen, kidney, thymus and the peritoneal cavity. They possess pseudopodia that they use for migration along the walls of blood vessels and through the tissue. Fish lymphocytes can be divided into two subpopulations: 1) surface immunoglobulin positive (sIg^+) lymphocytes that respond to lipopolysaccharide (LPS), resembling B cells in mammals; and 2) surface immunoglobulin negative (sIg^-) lymphocytes that respond to concanavalin A (Con A), resembling T cells in mammals. The sIg^+ and the sIg^- cells have been separated by an indirect "panning" technique, which uses monoclonal antibodies that recognize fish thymocyte or fish serum immunoglobulins [Christopher *et al.*, 1983; Secombes *et al.*, 1983; Sizemore *et al.*, 1984]. Antibody-producing lymphocytes have been found in the spleen and pronephros of salmonids and cyprinids [Anderson *et al.*, 1979; Bogner and Ellis, 1977; Rijkers *et al.*, 1980] but not in the thymus [Bogner and Ellis, 1977].

A number of investigators have examined the functions of fish lymphocytes *in vitro*: mitogenic responses, mixed lymphocyte reactions and antibody production [Clem *et al.*, 1984; Kaastrup *et al.*, 1988; Kaattari and Yui, 1987]. Carp peripheral blood leukocytes (PBL) respond *in vitro* to a variety of phytomitogens. The lymphocyte responses to Con A and phytohemagglutinin (PHA) are highly dependent on the continuous presence of mitogen in the medium. In contrast, a single stimulus with LPS, at the beginning of incubation, induces lymphocytes to proliferate for several days [Caspi and Avtalion, 1984]. Lymphokine production and plaque-forming cell assays to detect immunoglobulin M (IgM), have also been used to measure the immunological competence of fish lymphocytes [Graham and Secombes, 1990; Kaattari and Irwin, 1985].

Granulocytes: Three types of granulocytes have been found in fish blood. Type 1 fish granulocytes have two morphological forms: one has large eosinophilic granules with a nucleus that is irregular in shape yet rarely polymorphic; the other has much smaller granules with more neutrophilic character. This is the only granulocyte type ever seen with engulfed carbon and is capable of adhering to and spreading on glass or plastic, suggesting that it may be an analogue of the mammalian neutrophil [Parish *et al.*, 1985]. Further research showed that this granulocyte, like the neutrophils of mammals, is phagocytic and pinocytotic [Finco-Kent and Thune, 1987; Siwicki and Studnicka, 1987].

Type 2 granulocytes have slightly elongated, rod shaped granules that generally stain lighter than type 1 granulocytes. The nucleus of these cells is invariably polymorphic. Although adherent, these cells are not phagocytic and resemble, both functionally and morphologically, mammalian eosinophils [Parish *et al.*, 1985]. Ellis [1985] reported that this granulocyte, from rainbow trout, degranulates after endotoxin stimulation releasing toxic proteins and enzymes such as histaminase.

Type 3 granulocytes, which are unique to fish, have granules that do not stain with Giemsa. They appear as very bright round cells with an eccentric, dumb-bell shaped nucleus. Microscopic examination reveals that these cells are highly vacuolated [Parish *et al.*, 1985]. The function of these granulocytes remains unclear.

Nonspecific Cytotoxic Cells : In mammals and birds, NK cells have been well studied and are recognized to play an important role in immunosurveilance. Nonspecific cytotoxic cells possess cytolytic properties analogous to mammalian NK cells, and have been found in several species of fish [Carlson *et al.*, 1985; Hinuma *et al.*, 1980]. Fish NCC also possess anti-protozoan cytotoxicity [Graves *et al.*, 1985]. The following NCC responses have been examined in channel catfish: requirements for target cell lysis [Graves *et al.*, 1984], parameters of target specificity [Evans *et al.*, 1984a], biophysical and biochemical properties of NCC affecting cytolysis of the target cells [Evans *et al.*, 1984b], target cell binding and recycling capacity of the NCC [Evans *et al.*, 1984c], metabolic requirements of lysis [Carlson *et al.*, 1985], and flow cytometric analysis of the NCC [Evans *et al.*, 1987]. By comparing fish NCC with mammalian natural killer cells, the results of these studies indicate that fish NCC are phylogenetically related to the nonspecific cytotoxic cells found in higher vertebrates.

Fish Molecules with Immunological Properties

Immunoglobulins: Immunoglobulin M was considered the only immunoglobulin in fish [Kobayashi *et al.*, 1982], but a second class of immunoglobulins was identified in the serum of a cartilaginous fish by Kobayashi and coworkers [1984]. This immunoglobulin molecule is a non-covalently associated dimer with a sedimentation coefficient of 8.9S and a molecular mass of 320 kD. Unlike the pentameric IgM in mammals, IgM in most teleost fish is a tetrameric molecule composed of 8 heavy chains and 8 light chains (8H-8L) with a molecular mass of 600 to 800 kD [Håvarstein *et al.*, 1988; Lobb and Clem, 1983]. All fish IgMs seem to have a heavy chain similar to the mammalian μ-chain [Frommel *et al.*, 1971]. The fish immunoglobulins can opsonize foreign particles [Liewes *et al.*, 1982; Nash *et al.*, 1987], neutralize toxic materials produced by pathogens [Isbell and Pauley, 1983], form antigen-antibody complexes and activate complement [Ourth and Wilson, 1981; 1982a; 1982b].

Complement: Complement is a group of serum protein components involved in both specific and non-specific defenses. In mammals the complement system consists of a series of at least 18 proteins (including C_1-C_9) which can be activated in two ways. In the classical pathway, complement is activated by immune complex. In the alternative pathway, complement activation is accomplished by contact with bacterial cell wall polysaccharides or by aggregated IgA or IgG4.

Basic properties of mammalian complement (thermolability, requirement of Ca^{2+} and Mg^{2+}) are shared by fish complement. However, the temperature range over which complement remains active is far greater in fish. For example, fish complement retains its haemolytic activity at 0°C to 4°C. Six complement components have been isolated from nurse shark (*Ginglymostoma cirratum*) serum [Jensen *et al.*, 1981]. Three of these show compatibility with three of the nine components of mammalian complement. The fifth component of rainbow trout, *Salmo gairdneri*, complement was purified to homogeneity using a 4-step purification procedure. The highly purified C_5 retained its functional activity. The fraction C_5 can be cleaved into C_{5a} that is a phagocyte chemoattractant and C_{5b} that binds avidly to membranes and initiates the formation of the membrane attack complex [Nonaka *et al.*, 1981a]. The complement system can cause and/or participate in opsonization of foreign particles and thereby participate in

chemotaxis of neutrophils and macrophages, and initiate antibody-independent and antibody-dependent cell lysis [Rijkers *et al.*, 1980].

Lysozyme: Lysozyme, an enzyme with bacteriolytic properties because of its muramidase activity, is present in the serum and mucus of fish. Lysozyme is produced by phagocytic cells of many fish species including plaice (*Pleuronectes platessa* L.), channel catfish (*Ictalurus punctatus*), and carp (*Cyprinus carpio* L.) [Fletcher and White, 1973; Ourth, 1980]. The molecular mass of fish lysozyme, 15 kD, is similar to that of mammalian lysozyme, but a difference in electrophoretic mobility suggests differences in amino acid composition [Fletcher and White, 1973]. Variations in lysozyme activity between individual members within one species are considerable. After immunization of carp with *Aeromonas punctata*, the highest lysozyme activity coincided with the peak serum antibody concentrations [Murray and Fletcher, 1976]. Using immunohistochemical techniques, lysozyme activity could be demonstrated in monocytes and neutrophils suggesting that these cells produce lysozyme. Monocytes and neutrophils are probably the major source of the serum lysozyme, since the number of monocytes and neutrophils increase concomitantly with serum lysozyme levels after intravenous injection of latex beads [Murray and Fletcher, 1976].

C-reactive protein A: C-reactive protein (CRP) appears in mammalian serum during the acute phase of infection with microorganisms. CRP binds to phosphoryl choline residues that are present in the cell wall glycopeptides of various bacteria, fungi and on the surface of protozoan parasites. CRP can cause agglutination and precipitation of microorganisms and can activate the complement system. A serum component with CRP properties has been described for plaice, *Pleuronectes platessa* L. [Baldo and Fletcher, 1973]. In contrast to mammals, CRP in plaice is not an acute phase protein but a normal serum constituent that may provide fish with a permanent, non-specific (innate) defense against invading microorganisms. A protein that reacts with the C-polysaccharide of *Streptococcus pneumoniae* and is inhibited by phosphoryl choline, was isolated from the serum of rainbow trout by affinity chromatography. This protein is very similar to the CRP from rabbits. The possible value of fish CRP would be, first, as a modulator of immune reactions, and second, as a potent mediator of inflammatory responses [Winkelhake and Chang, 1982].

Natural hemagglutinins: Natural agglutinins have been detected from all classes of fish [Alexander, 1985]. The activity of agglutinins is usually directed against xenogenetic erythrocytes, more precisely towards carbohydrate moieties on the surface membranes of the cells. They share the following properties that distinguish them from immunoglobulins: (1) they are composed of identical subunits, so no distinction between heavy and light chains can be made; (2) no interchain disulfide bridges occur, the molecule is linked by noncovalent bonds; (3) upon electrophoresis no heterogeneity in amino acid composition of the subunits is displayed. A protein, consisting of 4 non-covalent linked subunits (90 kD), with agglutinating activity to human erythrocyte "O" antigen has been demonstrated in lamprey serum [Litman *et al.*, 1970]. In rainbow trout, natural hemagglutinins against rabbit, mouse and human erythrocytes were identified and characterized.

B. Immunoregulatory Properties of Glucocorticoids and Sex Hormones

In addition to exerting their important physiological effects on cell metabolism, activation and differentiation in the endocrine system, steroids also affect the function of cells of the immune system [Besedovsky *et al.*, 1985]. Accumulating evidence indicates that steroids, including both adrenal and gonadal steroids, modulate immune response in vertebrates including teleosts [Schuurs and Verheul, 1990; Slater and Schreck, 1993]. Glucocorticoids are the most intensively studied adrenal steroids for their immunosuppressive and anti-inflammatory effects [Cronstein *et al.*, 1992; Garvy and Fraker, 1991; Gatti *et al.*, 1987]. The effects of glucocorticoids on the functions of leukocytes are mainly suppressive. Most of the glucocorticoids, including both physiological and synthetic, inhibit a variety of functions of T and B lymphocytes [Garvy and Fraker, 1991; Bertoglio and Leroux, 1988], NK cells [Fuggetta *et al.*, 1988], macrophages [Fitzke and Dieter, 1991], and all three types of granulocytes [Charlensworth *et al.*, 1991; Howe *et al.*, 1990; Maloff *et al.*, 1989].

Differences in immune responses between female and male humans and animals have been known for a long time, but research in immunoregulatory properties of sex steroids has only been conducted during the past 15 years [Schuurs and Verheul, 1990]. Scientific interest in this "interdiscipline" between reproduction and immunity started with the accumulation of evidence in correla-

tion between sex hormone levels and immune responses. Research has since focused on the direct effects of sex steroids on leukocyte functions both *in vitro* and *in vivo* [Schuurs and Verheul, 1990]. However, as indicated by Schuurs and Verheul [1990], "Scientific knowledge regarding the influence of gender and sex hormones on the immune response in humans is on the one hand considerable but on the other hand disappointingly incoherent".

Immunoregulatory Properties of Glucocorticoids

A large body of literature indicates that glucocorticoids have suppressive effects on the immune responses in mammals. The immunosuppressive effects of glucocorticoids were initially observed in animals under stress, during which higher concentrations of glucocorticoids appear in the blood and the animals become more susceptible to infectious diseases [Stein *et al.*, 1985]. Intensive studies have revealed that stress-related immunosuppression is attributed to the suppressive effects of glucocorticoids on leukocytes [Dietch and McIntyre-Bridges, 1987; Stein *et al.*, 1985]. It is well known that glucocorticoids influence the function of all kinds of leukocytes including some hybridoma and leukemia cell lines [Aebischer and Schlegel-Haueter, 1992; McConkey *et al.*, 1991].

The Effects of Glucocorticoids on Lymphocytes: Glucocorticoids exert their immunosuppressive and anti-inflammatory actions by inhibiting lymphocyte functions such as proliferation, lymphokine production, receptor expression and immunoglobulin synthesis. For example, glucocorticoids inhibit the mitogen induced proliferation of human lymphocytes both *in vitro* and *in vivo* [Pukhalsky *et al.*, 1990; Rupprecht *et al.*, 1991], and inhibit the production of lymphokines such as IL-4, with suppression seen at both transcriptional and translational levels [Wu *et al.*, 1991]. Prednisolone suppresses both the spontaneous and IL-2- and IL-4-induced CD23 (Fc ε RII) expression, soluble CD23 release and synthesis of immunoglobulin by human peripheral blood lymphocytes [Fischer and Koenig, 1990]. The inhibition of IL-4 production and CD23 expression may explain, in part, the immunosuppressive effects of glucocorticoids in the treatment of allergic diseases [Katira *et al.*, 1993].

The Effects of Glucocorticoids on NK Cells: Glucocorticoids have suppressive effects on the functional activity of NK cells. Cortisol inhibits the NK activity of human peripheral blood mononuclear cells, as measured *in vitro* using a

direct ^{51}Cr-release assay and the K562 cell line as a target [Gatti *et al.*, 1987]. This inhibitory effect may be responsible for the higher susceptibility of glucocorticoid treated patients to viral infection and tumor development. The inhibitory effects of glucocorticoids on NK activity are modulated by IFNβ. Alternating the administration of IFNβ and glucocorticoids, in scheduled alternation, may override the suppressive effects of glucocorticoid therapy on natural immunity [Fuggetta *et al.*, 1988]. More recent research using a cloned human NK cell line demonstrated that cortisol at subphysiological concentrations directly inactivates the cytotoxic action of the NK cells without influencing their proliferation suggesting that the cytotoxic action of NK cells is extremely sensitive to glucocorticoids [Callewaert *et al.*, 1991].

The Effects of Glucocorticoids on Macrophages: Glucocorticosteroids inhibit a variety of macrophage functions including phagocytosis, anti-tumor activity, cytokine production, nitric oxide synthesis, and surface marker/receptor expression. For example, glucocorticoids inhibit tumoricidal activity of macrophages by inhibiting the activation of macrophages [Hogan and Vogel, 1988]. This inhibition of tumoricidal action may be mediated by inhibiting the IFNγ- and endotoxin-induced TNF synthesis by macrophages [Luedke and Cerami, 1990]. Cortexolone inhibits the production of nitrite and nitric oxide by the macrophage cell line J774 stimulated with LPS. The authors postulate that part of the anti-inflammatory and immunosuppressive actions of glucocorticoids may be due to their inhibition of the induction of the nitric oxide synthase [Di-Rosa *et al.*, 1990]. Moreover, dexamethasone inhibits the accumulation of macrophages in Con A-induced peritonitis in mice. This finding indicates that glucocorticoids inhibit the migration of macrophages postulating another possible anti-inflammatory mechanism for glucocorticoids [Nagaoka *et al.*, 1988].

Glucocorticoids inhibit the production of IL-1α and IL-1β by human peripheral blood mononuclear adherent cells stimulated with LPS. The data suggest that the suppression of IL-1 activity by glucocorticoids is consistently associated with decreased levels of IL-1 protein and mRNA expression [Lew *et al.*, 1988]. Further research suggests that this inhibition is mediated by decreased IL-1 mRNA stability [Amano *et al.*, 1993]. Dexamethasone inhibits IL-1 and IL-6 mRNA expression by human monocytes stimulated with LPS and this inhibition is abolished by the steroid receptor antagonist RU486. The authors suggest that the inhibition of multiple cytokine production by glucocorticoids may explain, in part,

the mechanisms of immunosuppressive effects of glucocorticoids [Amano *et al.*, 1993].

In macrophages, glucocorticoids inhibit the zymosan-induced formation of inositol phosphates, the intracellular second messengers responsible for cell activation, but do not inhibit the activity of phospholipase C [Fitzke and Dieter, 1991]. Cortisol inhibits the differentiation of human lymphoma cell line U937 into macrophage-like cells stimulated by PMA and this inhibition is reversible upon removal of the steroid. Cortisol also inhibits the IL-1 mRNA expression of the differentiated macrophages after stimulation with LPS [Baybutt and Holsboer, 1990].

The Effects of Glucocorticoids on Granulocytes: Glucocorticoids affect the functions of all three types of granulocytes. Dexamethasone inhibits the induction of Fc γ RI receptors on human neutrophils by IFNγ and the IFNγ stimulation of antibody-dependent cytotoxicity and phagocytosis of human neutrophils [Petroni *et al.*, 1988]. Dexamethasone inhibits the inducible nitric oxide synthase in rat peritoneal neutrophils and this inhibition may contribute to the anti-inflammatory activity of glucocorticoids [McCall *et al.*, 1991]. Glucocorticoids induce eosinopenia *in vivo* and inhibit cytokine-mediated survival of eosinophils [Hallsworth *et al.*, 1992; Wallen *et al.*, 1991]. Dexamethasone inhibits the response of eosinophils to granulocyte-macrophage colony-stimulating factor. The inhibitory effects of glucocorticoids on eosinophils may contribute to the anti-allergic action of the steroids [Lamas *et al.*, 1991]. Glucocorticoids also inhibit the IgE-dependent release of histamine by human basophils through a specific glucocorticoid receptor [Schleimer *et al.*, 1982]. The inhibitory effects of dexamethasone on human basophil mediator release is overridden by increased levels of IL-3, suggesting that the effects of glucocorticoids on human basophils may be, in part, mediated indirectly by affecting the cells that produce cytokines such as IFN γ and IL-3, which modulate basophil function [Schleimer *et al.*, 1989].

The Effects of Glucocorticosteroids on Teleost Immune Response: Glucocorticoids increase the susceptibility of fish to infectious diseases by inhibiting the normal function of macrophages and lymphocytes [Grimm, 1985; Pickering and Duston, 1983]. Cortisol reduces the mitogenic responses of coho salmon, *Oncorhynchus kisutch*, lymphocytes derived from the spleen and anterior kidney. The ability of pronephric lymphocytes to produce an antibody response

after the steroid treatment is restored by addition of supernatants from antigen stimulated lymphocyte cultures, suggesting that this cortisol-induced suppression may be mediated by inhibition of lymphokine production [Tripp *et al.*, 1987]. Cortisone depletes the lymphocyte populations in the lymphoid organs of rainbow trout and causes histologic lesions in the thymus [Chilmoczyk, 1985]. Cortisol can reduce the frequency of precursor lymphocytes in coho salmon [Kaattari and Tripp, 1987], and also reduce the normal ability of striped bass (*Morone saxatilis*) phagocytes to generate a chemiluminescence response when exposed to bacteria or phorbol 12-myristate acetate (PMA) [Stave and Roberson, 1985].

The Immunoregulatory Properties of Sex Steroids

Female and male animals show differences in heteroimmune and autoimmune responses. The mechanisms of the differences may be attributed to the immunoregulatory properties of sex steroids [Grossman, 1985]. Both female and male sex hormones modulate the functions of leukocytes, but their immunoregulatory effects on the immune system are complex. Both stimulatory and suppressive effects of sex steroids on immune response, and dose-dependent response of leukocytes to some sex steroids have been reported [Schuurs and Verheul, 1990]. Differential effects of sex steroids on humoral and cell-mediated immune responses have been demonstrated in a number of species. For example, estradiol inhibits the activity of cytotoxic T cells but enhances immunoglobulin production by B cells [Grossman, 1985]. Generally speaking, androgens and progestins are immunosuppressive while estrogens are immunosuppressive at pharmacological concentrations and immuno-stimulatory at physiological concentrations [Schuurs and Verheul, 1990].

The Effects of Androgens on Immune Response: The constantly lower immune response in males compared to females indicates that the male sex hormone androgens may have immunosuppressive properties in mammals [Grossman, 1985]. Endogenous testosterone, induced by administration of human chorionic gonadotropin to prepubertal boys with incomplete descent of one testis, causes a rise in testosterone to normal levels together with a significant decrease in the $CD4^+/CD8^+$ ratio, suggesting that testosterone may regulate immune responses by affecting the differentiation and maturation of T lymphocytes [Dunkel *et al.*, 1985]. A more recent study showed that orchidectomy and testosterone replacement alter the immune response of mice to T-dependent and T-independent

antigens [Rife *et al.*, 1990]. This alteration is determined by examining antibody-producing cells and testosterone receptors in spleen lymphocytes. The results showed that testosterone inhibits the immune response of mice to T-dependent antigens and that no testosterone receptors were observed in the spleen lymphocytes suggesting that testosterone regulates the immune system through the enhancement of suppressive activity of T-lymphocytes [Rife *et al.*, 1990]. Testosterone administration increases the susceptibility of mice to a self-healing malaria *Plasmodium chabaudi* and alters the protein expression in functionally changed splenic non-T cells [Schmitt-Wrede *et al.*, 1991]. Testosterone also inhibits the leukocyte functions *in vitro*. At physiological concentrations (10 to 300 ng/ml), testosterone inhibits the differentiation of human B lymphocytes after pokeweed mitogen stimulation determined by the plaque-forming cell assay [Sthoeger *et al.*, 1988].

The Effects of Progestins on Immune Response: The immunosuppressive effects of progestins were initially observed in women and animals during pregnancy, when progesterone appeared in high levels in their blood [Schuurs and Verheul, 1990]. The pregnancy-related suppression in immune responses includes decreased mitogenic response and cell-mediated immunity, suppressed NK cell activity, and prolonged homograft survival [Schuurs and Verheul, 1990]. Progesterone modifies uterine immune function by regulating the migration or proliferation of endometrial lymphocyte populations [Gottshall and Hansen, 1992]. Progesterone inhibits the cytotoxicity of human peripheral blood mononuclear cells and this suppression is synergistically enhanced by estradiol, estrone, and estriol [Feinberg *et al.*, 1992]. The inhibitory action of progesterone and estradiol may contribute to fetal survival since progesterone and estradiol secreted by trophoblasts, act at the choriodecidual interface where the steroids are present in high concentrations to provide a local, paracrine immunosuppressive effect on cellular cytotoxicity [Feinberg *et al.*, 1992]. Progesterone and estradiol inhibit IL-1β mRNA expression by human peripheral blood monocytes. This reciprocal relationship between IL-1 and gonadal steroids may have important ramifications in reproductive biology for both embryonic implantation and fetal survival [Polan *et al.*, 1989]. The demonstration of progesterone receptors in lymphocytes provides a molecular basis for the immunosuppressive effects of progesterone [Szekeres-Bartho *et al.*, 1989a; 1989b].

The Effects of Estrogens on Immune Response: In general, females have consistently higher immunoglobulin levels than males and mount higher antibody responses to microorganisms, suggesting that female sex steroids may have stimulatory effects on B lymphocytes [Schuurs and Verheul, 1990]. Estradiol at physiological concentrations increases granulocyte-macrophage colony formation from human peripheral blood monocytes [Barak *et al.*, 1986]. Macrophages from adult female rats secret greater amounts of IL-1 than those from age-matched males suggesting that estradiol may play an important role in regulating synthesis of IL-1 by macrophages [Hu *et al.*, 1988]. Physiological levels of estradiol enhance *in vivo* humoral immunity and this enhancement is thymus-dependent. A constitutive thymic factor, existed in thymosin fraction 5, exerts a permissive influence on the action of estradiol [Erbach and Bahr, 1991].

However, estradiol at higher concentrations can suppress the immune responses. Estradiol administration increases the susceptibility and mortality of mice to disseminated gonococcal infection indicating that estradiol has immunosuppressive action *in vivo* [Kita *et al.*, 1985]. Estradiol, at prepartum concentrations, inhibits proliferation of lymphocytes and phagocytosis of granulocytes of porcine [Magnusson, 1991]. Estradiol also inhibits the expression of Class I and Class II MHC antigens on guinea pig blood leukocytes indicating that it may interfere with the antigen processing function by antigen-presenting cells [Debout *et al.*, 1991].

The Effects of Sex Steroids on Teleost Immune Response: Little is known about the immunoregulatory action of sex steroids in fish. Only testosterone has been reported to inhibit the function of antibody producing cells in chinook salmon, *Oncorhynchus tshawytscha* [Slater and Schreck, 1993]. Clearly, more research is needed to define immunoregulatory properties of sex hormones and their effects on the susceptibility of fish to infectious diseases.

Steroid Receptors in Leukocytes:

All steroids affect their target cells via a group of intracellular receptors belonging to the steroid receptor superfamily [Reichel and Jacob, 1993]. In addition to the classic target cells, steroid receptors have been found in all types of leukocytes including T and B lymphocytes [Armanini *et al.*, 1988], macrophages [Salkowski and Vogel, 1992], and granulocytes [Prin *et al.*, 1989]. Interestingly, the expression of glucocorticoid receptors in glucocorticoid sensitive leukocytes

was up-regulated by glucocorticoids [Eisen *et al.*, 1988]. This finding suggests a positive feed-back mechanism for glucocorticoids to exert their immunosuppressive effects [Eisen *et al.*, 1988]. However, the expression of glucocorticoid receptor mRNA in glucocorticoid resistant leukocytes was down-regulated by the steroids [Rosewicz *et al.*, 1988], indicating that this negative feed-back mechanism may attribute to the reduction of responsiveness of some allergic patients to steroid therapy.

In fish, glucocorticoid receptors have been found in leukocytes of juvenile coho salmon, *Oncorhynchus kisutch* [Maule and Schreck, 1990]. The expression of these receptors is down-regulated by stress or cortisol treatment [Maule and Schreck, 1991]. Seasonal changes in cortisol sensitivity and glucocorticoid receptor affinity and number in leukocytes of coho salmon have been demonstrated by Maule and colleagues [1993].

The Mechanisms of Induction of Immunosuppression by Steroids:

The mechanisms of induction of immunosuppression by steroids are not clear. The following are hypotheses of potential mechanisms governing immunosuppression by steroids:

(1). Induction of Apoptosis: Glucocorticoids induce apoptosis in human T cell-derived leukemia cell lines [Bansal *et al.*, 1991], murine bone marrow B-lineage lymphocytes [Garvy *et al.*, 1993], and immature murine T cells [Gruol and Altschmied, 1993]. The steroid-induced apoptosis has the common characteristic feature of apoptosis induced by other factors such antigen- or mitogen-induced activation of immature lymphocytes, i.e. fragmentation of DNA at internucleosomal linkers through the activation of a specific endonuclease [Bansal *et al.*, 1991]. Glucocorticoids also induce apoptosis in mature T helper (Th) cells. This steroid-induced apoptosis is inhibited by addition of IL-2 and IL-4, which selectively rescues Th 1 and Th 2 subsets, respectively. The *in vitro* data show that mature T cells can be protected, by their own growth factors from the deleterious effects of glucocorticoids. This suggests that specific interactions may occur between lymphokines and naturally produced glucocorticoids *in vivo*, that may play a role in the regulation of immune responses [Zubiaga *et al.*, 1992].

(2). Inhibition of Growth Factor Production: Glucocorticoids inhibit the production of multiple cytokines and growth factors such as IL-2 [Vacca, *et al.*,

1992], IL-1 and IL-6 [Amano *et al.*, 1993], IL-4 [Byron *et al.*, 1992; Wu *et al.*, 1991], and IL-5 [Rolfe *et al.*, 1992]. Inhibition of cytokine production may explain, in part, the suppressive effects of glucocorticoids on immune responses. Some of the inhibition is mediated by interrupting the autocrine/paracrine actions of the cytokines.

(3). Influence the Metabolism of Leukocytes: The metabolism and function of macrophages are markedly inhibited by dexamethasone. The maximum activities of hexokinase, glucose-6-phosphate dehydrogenase, glutaminase and citrate synthase are significantly lower in macrophages obtained from hormone-treated rats and cultures incubated with the hormone for 48 hrs than those from untreated controls [Costa-Rosa *et al.*, 1992]. Dexamethasone also inhibits macrophage phagocytosis and this inhibition is altered when insulin is added into culture medium. These data support the suggestion that the immunosuppressive and anti-inflammatory effects of glucocorticoids are mediated through changes in macrophage metabolism [Costa-Rosa *et al.*, 1992]. Stress or dexamethasone administration reduce the rate of pyruvate utilization and increase the rate of lactate production by rat lymphocytes as compared to control rats [Serrano and Curi, 1992]. These findings suggest that glucocorticoids exert their suppressive effects on immune responses through regulating metabolism in lymphocytes [Serrano and Curi, 1992].

In conclusion, glucocorticoids cause suppression of the functions of T and B lymphocytes, NK cells, macrophages and granulocytes. Sex steroids have suppressive or stimulatory effects on immune response. In general, androgens and progestins are immunosuppressive, while estrogens are immunostimulatory at physiological concentrations and immunosuppressive at pharmacological concentrations. Steroids exert their immunoregulatory actions through a group of intracellular receptors belonging to the steroid receptor superfamily. They induce apoptosis, inhibit cytokine/growth factor production and interfere with the metabolism of leukocytes.

C. *Trypanosoma danilewskyi* INFECTION IN GOLDFISH

Trypanosoma danilewskyi Laveren and Mesnil (1904) is a common carp, *Cyprinus carpio*, haemoflagellate that naturally occurs in Europe and the former USSR [Laveran and Mesnil, 1907; Pavlovskii, 1964]. *T. danilewskyi* is also infec-

tive to other cyprinid fish such as the crucian carp *Carassius auratus gibelio* [Woo, 1981a]. The intermediate host in the life cycle is a glossophonoid leech (*Hemiclepsis marginata*). The haemoflagellate is ingested by the leech as it feeds on an infected fish and reaches the leech's crop within about three hours. In the crop of the leech, the trypanosomes change to short, stumpy forms within 24 hrs and multiply for several days. The haemoflagellates develop into the infective metatrypanosomes in about 10 days. They migrate from the crop into the proboscis sheath and are transmitted to the new definite host when the leech feeds [Qadri, 1962]. Woo [1981a] described the four-stage multiplication process for *T. danilewskyi*: (a) the production of a new flagellum followed by kinetoplast division; (b) the production of new cytoplasm around the posteriorly flipped new flagellum; (c) division of the nucleus and migration of one of the nuclei toward the posterior; and (d) division of the body.

In 1973, Lom established *T. danilewskyi* infections in goldfish by intraperitoneal inoculation of fish blood containing the trypanosomes. Eighty percent of experimentally infected goldfish died after inoculation. The surviving fish eliminated the parasites by day 48 of infection and developed resistance to reinfection [Lom, 1973a; 1973b]. Woo [1981b] reported that previously infected goldfish were protected from re-infection for up to 190 days after the primary infection. In addition, this protection can be passively transferred to naive hosts with immune plasma. Interestingly, trypanosomes incubated for 2 hours at 20°C with the immune plasma lose their infectivity, indicating that specific antibodies and/or other infection blocking molecules in the plasma play a major role in this protection. No lysis of trypanosomes has been observed *in vitro* by the immune plasma [Woo, 1981b].

To date, no attempts have been made to examine the cell-mediated immunity in this host-parasite association. The precise mechanisms of host defense against *T. danilewskyi* in goldfish remain to be defined. An understanding of the host defense mechanisms against this haemoflagellate and the regulation of protective immune responses will provide valuable information for the potential prevention of infectious diseases in fish.

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Chapter Three: Development of Immunological Assays for Goldfish Lymphocytes and Macrophages

INTRODUCTION

Lymphocytes and macrophages are two major participants in specific immune responses against infectious diseases in fish [Chung and Secombes, 1988; Kaattari and Irwin, 1985]. Like higher vertebrates, fish have two distinct populations of lymphocytes: thymus-independent lymphocytes (sIg^+ or B lymphocytes) and thymus-dependent lymphocytes (sIg^- or T lymphocytes). Fish sIg^- lymphocytes secrete molecules with interferon-like activities [Graham and Secombes, 1988]; thus, they may participate in the regulation of immune responses. Mitogen-induced lymphocyte proliferation has been used to evaluate the lymphocyte function of several fish species including channel catfish [Miller and Clem, 1988], trout [Kaattari *et al.*, 1985], carp [Caspi *et al.*, 1984], and plaice [Grimm, 1985].

Macrophages participate in both non-specific and specific immune responses by executing their biological activities such as chemotaxis, phagocytosis, cytotoxicity, nitric oxide synthesis, cytokine production, and respiratory burst. They also produce a number of protein molecules such as C-reactive protein A and lysozyme, which participate in the inflammatory and antimicrobial responses [Fletcher and White 1973; Ourth 1980]. Macrophages facilitate specific immune responses by performing their antigen presenting function. They engulf antigen particles, enzymatically break down antigen macromolecules and present antigenic determinants to lymphocytes [Vallejo *et al.*, 1992]. Phagocytosis and production of reactive oxygen intermediates have been reported in trout and carp macrophages [Avtalion and Shahrabani, 1975; Chung and Secombes, 1987; 1988; Olivier *et al.*, 1986]. The chemotaxis of fish macrophages has been reported in trout [Griffin, 1984], carp [stG Howell, 1987], plaice [MacArthur *et al.*, 1985], and nurse shark [Obenauf and Hyder Smith, 1985].

In my attempts to examine the immunoregulatory effects of the steroids estradiol and cortisol on goldfish immune responses, I required immunoassays for evaluation of the functions of goldfish lymphocytes and macrophages after treatment with steroids. In this chapter, I describe the development of immunological assays for goldfish lymphocytes and macrophages, which are used throughout this thesis.

MATERIALS AND METHODS

Fish:

Goldfish were purchased when 4 to 5 cm long from either Ozark Fisheries Inc. (Southland, MI) or Grassy Forks Fisheries (Martinsville, IN) and maintained at the Aquatic Facility of the Department of Zoology, University of Alberta. The fish were held at 20°C in a flow-through water system on a simulated natural photoperiod (Edmonton, Alberta) for the duration of the experiments and fed to satiation daily with trout pellets. They were acclimated to this environment for 3 weeks before use.

Culture Media for Goldfish Lymphocytes and Macrophages:

A culture medium, designated as Goldfish Leukocyte Medium (GFLM), for goldfish lymphocytes and macrophages was developed. The composition of the medium is specified in Table 3-1. The GFLM is based on the commercial media Leibovitz's L-15 and Dulbecco's Modified Eagle (GIBCO, Grand Island, New York, USA) and additional nutrients (Table 3-1). Because the cells were cultured in an environment without carbon dioxide, the amount of sodium bicarbonate was reduced and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was added to increase the buffering capacity of the medium. The initial pH of the medium was adjusted to 7.2 at room temperature using 1N NaOH. The incomplete medium (without serum) was designated as wash medium and used to dilute fish blood, and to wash fish organs and cells. The completed medium (culture medium) was supplemented with 10% bovine calf serum (Hyclone, Logan, Utah; Cat. No. A-2151) and 5% goldfish serum obtained from a group of healthy donors. The incomplete and complete media were stored at 4°C for up to 30 days.

A. MITOGEN-INDUCED LYMPHOCYTE PROLIFERATION:

Isolation of Goldfish Peripheral Blood Lymphocytes:

Goldfish (50 to 100 g body weight) were anesthetized with 0.05% tricaine methanesulfonate (MS222; Syndel Laboratories Ltd. Vancouver, B. C), and 1 to 2 ml of blood were taken from the caudal vein using a heparinized syringe fitted with a 23 gauge needle. The blood was diluted with the wash medium (1 : 2) and

washed once by centrifugation at 300 x *g* for 10 min. at 4°C. The cells were resuspended in 4 to 6 ml of wash medium, carefully layered onto 4 ml 100% Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscatway, NJ) in polypropylene tubes, and centrifuged at 350 x *g* for 24 min. at 4°C. The leukocytes (containing 60 to 70% lymphocytes) at the Ficoll-medium interface were transferred to 15 ml centrifuge tubes and washed 3 times by centrifugation at 300 x *g* for 10 min. at 4°C. The cells were resuspended in 10 ml of culture medium, enumerated using a haemocytometer and their number was adjusted to 2.5 x 10^6 per ml.

Proliferation Assay:

Goldfish PBL were placed in each well of a sterile 96 well microtiter plate (5 x 10^5 cells in 200 µl). In the preliminary experiments, the working concentrations of PMA and A23187 were determined by examining a wider range of concentrations for the chemicals (PMA: 5 ng/ml to 1000 ng/ml; A23187: 25 to 10 µg/ml). The proliferative responses of the PBL were determined after treatment of the cells with PMA (Sigma, 25 ng per ml to 200 ng per ml) plus calcium ionophore (A23187; Sigma, 125 ng per ml to 1000 ng per ml), and PMA or A23187 only. The plates containing PMA and A23187 were incubated at 20°C for 12 hrs. After incubation, the medium in the wells was replaced since PMA and A23187 are toxic to the cells [Miller and Clem, 1988]. The plates were reincubated at 20°C for 84 hrs. Two types of controls, medium containing cells without DMSO-mitogens and medium containing cells and DMSO without mitogens were used in the experiments.

To determine the optimum concentrations for PMA, A23187 and their combinations, dose response experiments were done. In the first set of experiments, the concentration of PMA was constant at 100 ng per ml and different concentrations of A23187 were used (125 to 1000 ng per ml). In the second set of experiments, the concentration of A23187 was constant (500 ng per ml) and that of PMA was from 25 to 200 ng per ml.

The proliferation of goldfish PBL was measured using [^{3}H]thymidine incorporation. After a total of 96 hrs of incubation, the cells were pulsed (treated) with 2 µCi per well [^{3}H]thymidine (New England Nuclear, Boston, MA) and reincubated at 20°C for 16 hrs. The cells were harvested onto glass microfibre filters (Type GF/A; Whatman International Ltd. Maidstone, England) by lysing them with 0.1% sodium dodecyl sulfate (SDS; Bethesda Research Laboratories, MD.) in

Milli-Q water. The filters were air dried , placed into scintillation vials filled with 6 ml of scintillation fluid (ScientiVerse™, Fisher Scientific Co., Fair Lawn, NJ), and counted using a scintillation counter (L1217, Pharmacia LKB Biotechnology, Uppsala, Sweden).

B. ASSAYS FOR MACROPHAGE FUNCTION:

Macrophages:

Macrophages were isolated from the kidneys of goldfish. The fish were anesthetized with MS222, bled, and killed. The kidneys were removed aseptically, washed 4 times with ice-cold wash medium, and pressed through a 50 mesh per cm^2 sterile stainless steel screens. The cells were washed by centrifugation at 300 x *g* for 10 min. at 4°C and resuspended in wash medium. For purification of macrophages, the cell suspension was layered on a 34%-51% Percoll (Sigma Chemical Co., St. Louis. MO) discontinuous gradient and centrifuged at 350 x *g* for 24 minutes [Chung and Secombes, 1988; Secombes *et al.*, 1988]. The cells at the 34%-51% Percoll interface were transferred into clean tubes and washed 3 times by centrifugation at 300 x *g* for 10 min. at 4°C. Purified macrophages were enumerated with a haemocytometer and suspended at a concentration of 10^6 per ml prior to use in the assays.

Macrophage Cell Line:

A goldfish kidney macrophage cell line was established and maintained in culture for 2 years. The cultured macrophages grow spontaneously in culture. The morphology of the cells was examined using both light microscope (LM) and transmission electron microscope (TEM). For bright field LM observations, the cells were centrifuged to make slides using a cytocentrifuge (Shandon Southern Products Ltd. Astmoor, England) at 700 rpm for 7 min. and stained using LeukoStat™ Solutions (Fisher Scientific Co. Fair Lawn, NJ). Photographs were taken under oil immersion using a Nikon MICROPHOT-FXA microscope. For TEM observations, the cells were prefixed in buffered (0.1 M cacodylate buffer, pH 7.2) 2.0% glutaraldehyde (Sigma) at room temperature for 2 hrs and fixed in buffered (0.1 M cacodylate buffer, pH 7.2) 2.0% osmium tetroxide (Sigma) at room temperature for 1 hr. The fixed cells were dehydrated using gradient concentrations of ethanol and embedded using Epon (Sigma). Ultra-thin sections were

made using an Ultracut E (Reichert-Jung, Austria) microtome and the sections were observed and photographed using a Philips T400 TEM.

For karyotype analysis the chromosomes were prepared using the procedure reported by Blaxhall [1983]. The cells were incubated with 5 µg per ml colchicine in culture medium at 20°C for 6 hrs, treated with 0.4% KCl for 10 min. and fixed with methanol : acetate acid solution (3 : 1, freshly prepared). The chromosomes were spread by dropping the cells onto slides and stained with Giemsa solution.

The Assays:

(a). Chemotaxis: A modified chemotaxis assay described by Belosevic and Faubert [1986] was used to examine the chemotaxis of goldfish macrophages. Endotoxin activated goldfish serum (EAGFS) was used as the chemoattractant. Activation of complement by bacterial endotoxin generates the anaphylatoxins C3a and C5a, the most important complement-derived chemoattractants [MacArthur *et al.*, 1985]. Goldfish serum was activated with endotoxin and was investigated for its ability to attract fish macrophages. Briefly, EAGFS was prepared by mixing 1 ml of goldfish serum with 2 mg LPS. The mixture was incubated at 27°C for 2 hours, placed in a 56°C water bath for 30 minutes, and centrifuged at 20,000 x *g* for 30 minutes at 4°C. The supernatant (designated as EAGFS) was collected and stored at -20°C until use. The bottom chambers of the Neuro Probe chemotaxis unit (Nuclepore Corporation, Pleasanton, CA) were filled with 200 µl of culture medium containing different dilutions of EAGFS (1 : 50 to 1 : 400). A Nuclepore chemotaxis membrane (Nuclepore Corporation, Pleasanton, CA; 5 µm pore size) was placed over the bottom chamber and the top chamber screwed on. The upper chamber was filled with 5 x 10^4 goldfish macrophages in 200 µl of culture medium. The chambers were then incubated at 20°C for 2 hrs. After incubation, the upper chamber was emptied and dried with a cotton swab. The membrane was removed, put on a microscope slide (upside down), dried in the air and stained with Giemsa solution. The macrophages that migrated through the pores of the membranes and adhered on the underside of the Nuclepore membranes were enumerated using a microscope (100 X).

In parallel experiments, the bottom chamber was filled with culture medium containing different dilutions of EAGFS (1 : 50 to 1 : 400). A Nuclepore chemotaxis membrane was placed over the bottom chamber and the upper chamber screwed on. The upper chamber was filled with 2 x 10^5 cells in 200 µl culture

medium. The chambers were incubated at 20°C for 2 hrs, and the medium in the upper chamber and the membrane were removed. The cells that have migrated through the pores of the Nuclepore membranes and accumulated in the bottom chamber were enumerated using a haemocytometer.

(b). Phagocytosis: Sheep red blood cells (SRBC) were used to test the phagocytosis of goldfish macrophages. Briefly, 5 x 10^4 macrophages were incubated with 2.5 x 10^5 to 2 x 10^6 SRBC (1 : 5 to 1 : 40) at 20°C for 2 hours in polypropylene tubes. After centrifugation at 150 x *g* for 10 min, the supernatant was removed and the free SRBC were lysed using a sterile lysing buffer*. The macrophages were washed, centrifuged using a Cytospin 2 centrifuge (Shandon) at 700 rpm for 7 min. and stained with LeukoStat™ Solutions (Fisher). The numbers of macrophages with and without SRBC were enumerated by counting 200 macrophages and the percent of macrophages with engulfed SRBC calculated. To determine the phagocytic ability of individual macrophages, the number of SRBC per macrophage was determined by counting the number of SRBC in 50 macrophages.

To determine the opsonizing effects of antibody on the phagocytosis of goldfish macrophages, the SRBC were pretreated with different concentrations of rabbit anti-SRBC antibodies. The antibody-treated SRBC were used as targets in the phagocytosis assay.

(c). Nitric Oxide Production: To detect nitric oxide production of goldfish macrophages, the method for detecting nitric oxide production by murine macrophages was used [Green *et al.*, 1990]. Briefly, goldfish macrophages were seeded into 96-well microplates (5 x 10^5 per well) and activated with different concentrations of LPS. After 96 hours in culture, the cell-free culture supernatants were assayed for nitrite using the Griess reaction. Fifty microliter aliquots of the supernatant were incubated with 100 µl of 1% sulfanilamide and 100 µl of 0.1% N-1-naphthylethylenediamine dihydrochloride (Sigma) in 2.5% H_3PO_4 at room temperature for 5 minutes. Optical density was measured in a microplate reader (Model EL311, Bio-Tek Instruments Inc, Winooski, VT) at 570 nm and nitrite concentrations were quantified by comparison to $NaNO_2$ (Sigma) standards.

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* The lysing buffer consisted of 4.18 g ammonium chloride, 0.5 g potassium hydrogen carbonate, 0.02 g disodium ethylenediaminetetraacetic acid (EDTA) in 500 ml Milli Q® water.

(d). Respiratory Burst: The O_2^- production of goldfish macrophages was detected by a modified nitroblue tetrazolium (NBT) reduction assay originally described by Chung and Secombes [1988]. Macrophages were placed into wells of a 96-well microtiter plate (2.5 x 10^5 per well) and incubated at 20°C for 48 hrs. In one group of experiments, different concentrations of LPS (10 to 50 µg per ml) were added to the wells and the cells were reincubated for an additional 12 hrs. After removal of the old medium, the macrophage monolayer was covered with 100 µl of 1 mg per ml of NBT (Sigma) in culture medium containing 50 ng per ml of PMA. In other experiments, 20 µg per ml of LPS was added to the wells and the cells were incubated for an additional 12 hrs. The macrophage monolayer was then covered with 100 µl of 1 mg per ml of NBT in culture medium containing different concentrations of PMA (10 to 100 ng per ml). The plates with stimulated cells were incubated for 60 min. at 20°C. The reduced formazan within macrophages was solubilized in KOH and dimethyl sulphoxide (DMSO) after fixing and washing the cells with methanol. Optical density was measured using a microplate reader (Bio-Tek) at 630 nm.

RESULTS

Mitogen-induced Lymphocyte Proliferation:

Mitogen-induced proliferation is an important criterion for evaluating the function of lymphocytes. The [^{3}H]thymidine incorporation assay is the most commonly used method for determination of the mitogen-induced proliferative responses of lymphocytes. Co-stimulation with PMA and A23187 induced strong proliferation of goldfish lymphocytes. The count per minute (CPM) of [^{3}H]thymidine incorporation into goldfish PBL increased on the average by 10 fold when the lymphocytes were treated with the combination of PMA and A23187. However, when used individually only PMA induced significant proliferation of the cells, but at a lower level than in combination with A23187, indicating their synergistic interaction. In the no mitogen and solvent (DMSO) controls, the average CPM is less than 2000.

Two sets of dose response experiments were done to determine the optimum concentrations for PMA and A23187 (Fig. 3-1; Fig. 3-2). Figure 3-1 depicts the data obtained in PMA dose-response experiments: constant A23187

(500 ng per ml) with different amounts of PMA (25 to 200 ng per ml). The results showed that PMA, at all concentrations, induced significant proliferation of goldfish PBL (Fig. 3-1). The data obtained in A23187 dose-response experiments are presented in Figure 3-2, where constant PMA (100 ng per ml) and different amounts of A23187 (125 to 1000 ng per ml) were used. The data indicated that A23187 at all concentrations tested induced significant proliferation of the cells, although the CPM was lower when the cells were incubated with 1000 ng per ml of A23187 (Fig. 3-2).

The Characteristics of Goldfish Macrophage Cell Line:

Morphologically and functionally the cultured goldfish macrophages resemble mammalian macrophages: they are irregular in shape due to the formation of pseudopodia, have alkaline cytoplasm containing many vacuoles (Fig. 3-3A), are adherent to glass and plastic, and are actively motile. Observation with TEM showed that they have similar ultrastructure to mammalian macrophages: they possess pseudopodia and vacuoles, have mitochondria and endoplasmic reticulum in their cytoplasm, and their nuclei occupy less than 50% of the cells volume (Fig. 3-4A). Karyotype analysis showed that these cells have a tetraploid karyotype with 100 chromosomes in each cell indicating that they are normal goldfish cells (Fig. 3-4B)[Buth *et al.*, 1991]. The macrophage cell lines have the following physiological characteristics: they exhibit chemotaxis, engulf SRBC, synthesize nitric oxide in the presence of L-arginine after stimulation with LPS and produce O_2^- upon activation with PMA and LPS.

Chemotaxis of Goldfish Macrophages:

Goldfish macrophages showed active migration towards the chemoattractant EAGFS. Without the chemoattractant (control), an average of 80 ± 8 cells were found on the membrane per 100 microscopic oil fields (MOF). At 1 : 200 ($p = 0.02$), 1 : 100 ($p = 0.03$) and 1 : 50 ($p = 0.04$) dilutions of EAGFS, statistically significant increases in chemotaxis were observed (Fig. 3-5A). The average number of cells on the membrane increased to more than 116 ± 12 per 100 MOF.

In parallel experiments, the cells accumulated in the bottom chamber were enumerated using a haemocytometer. Without the EAGFS, $6.1 \pm 0.7 \times 10^4$ per ml of the cells migrated through the membrane and accumulated at the bottom of the chamber. At a 1 : 100 dilution of the EAGFS, $10.1 \pm 0.6 \times 10^4$ per ml of the cells

migrated through the membrane and entered the lower chamber. At 1 : 200 and 1 : 50 dilutions of the EAGFS, the numbers of cells migrated through the filter were 8 $\pm$ 0.6 x 10^4 per ml and 8.5 $\pm$ 0.4 x 10^4 per ml, respectively (Fig. 3-5B). Statistical analysis using a Student t-test indicated significant differences in chemotactic responses of goldfish macrophages between the control and a 1 : 50 ($p = 0.004$), the control and a 1 : 100 ($p = 0.0001$), the control and a 1 : 200 ($p = 0.022$), but not the control and a 1 : 400 ($p = 0.464$) dilution of the EAGFS.

Phagocytosis of Goldfish Macrophages:

The most common targets used in phagocytosis assay are foreign red blood cells (RBC). I used sheep RBC in the assay as targets for the assessment of the phagocytosis of goldfish macrophages. The results showed that goldfish macrophages actively engulfed SRBC (Fig. 3-3B). At the macrophage : SRBC ratio of 1 : 10, about 14% of the macrophages contained engulfed SRBC. The phagocytosis was higher when the ratio of SRBC to macrophage was higher. At the ratio of 1 : 20, about 24% of macrophages engulfed SRBC. The number of SRBC per macrophage also increased when more SRBC was used. At the ratio of 1 : 20, the average number of SRBC per macrophage was 2.27 $\pm$ 0.25 (Table 3-2).

Another advantage for using SRBC in phagocytic assays is the availability of anti-SRBC antibodies, which can be used to evaluate the opsonized phagocytosis of the cells. The number of macrophages having engulfed SRBC significantly increased when the SRBC were coated with rabbit anti-SRBC serum (Fig. 3-6A). The number of SRBC per macrophage also increased (Fig. 3-6B).The anti-serum at a 1 : 400 dilution caused a 15% increase in phagocytosis (about 40%) compared to the non-opsonized control (25%). At 1 : 200 and 1 : 800 dilution, the antiserum also induced a significant increase in phagocytosis of the cells. At 1 : 100 dilution, the antiserum caused about 6% decrease in phagocytosis. The serum at 1 : 400 dilution was optimal for opsonization while 1 : 100 dilution induced inhibition of phagocytosis. The inhibitory effect at higher concentrations of the antiserum may be due to the significant agglutination of the SRBC.

Nitric Oxide Production of Goldfish Macrophages:

Goldfish macrophages produce nitric oxide when stimulated with bacterial LPS (Fig. 3-7). The macrophages showed a dose dependent nitric oxide production in response to LPS at concentrations of 5 μg per ml and greater ($p < 0.05$ for all

groups, t-test). LPS at a concentration of 20 μg per ml showed optimal stimulation for synthesis of nitrite. The ability of goldfish macrophages to produce nitric oxide suggests that fish macrophages may use similar anti-microbial and anti-tumor molecules for killing of pathogens and tumors. The metabolic pathways of nitric oxide production in fish macrophages remain to be determined.

Respiratory Burst of Goldfish Macrophages:

The production of O_2^- was measured using NBT reduction assay [Graham and Secombes, 1988]. In the present study, goldfish macrophages produce O_2^- in response to PMA and LPS. The co-stimulation of macrophages with PMA and LPS resulted in much higher O_2^- production (Fig. 3-8A; 3-8B). Two sets of experiments were done. In the first set of experiments, a constant PMA concentration (50 ng per ml) and variable LPS concentrations (5 to 30 μg per ml) were used. LPS at concentrations of 15-20 μg per ml in the presence of PMA induced maximum O_2^- production (Fig. 3-8A). Statistical analysis using t-test showed significant differences between untreated control and all experimental groups ($p < 0.05$). In the second sets of experiments, a fixed LPS concentration (20 μg per ml) and variable PMA concentrations (12.5 to 100 ng per ml) were tested. PMA at concentrations of 25-75 ng per ml plus LPS at 20 μg per ml induced maximum O_2^- production (Fig. 3-8B). There were significant differences (t-test) between control (no mitogen) and all experimental groups ($p < 0.05$). When used alone, LPS at a concentration of 20 μg per ml or PMA at a concentration of 50 ng per ml induced a much lower, albeit significant, O_2^- production. Comparison of the O_2^- production induced by PMA or LPS alone to that induced by a combination of PMA and LPS showed a highly significant difference ($p < 0.001$), indicating a synergistic interaction between PMA and LPS for induction of respiratory burst of fish macrophages.

DISCUSSION

A competent immune system is essential for fish to survive in the challenging environment, where a variety of microorganisms including viruses, bacteria, fungi, and protozoa also make their living. The immune system also

protects fish from tumor development by executing its "surveillance" function [Kaattari and Irwin, 1985; Secombes *et al.*, 1983].

Mitogen-induced proliferation of lymphocytes has been reported in several fish species including channel catfish [Miller and Clem, 1988], salmonids [Kaattari and Yui, 1987], carp [Caspi *et al.*, 1984], and plaice [Grimm, 1985]. To date, the mitogen-induced proliferation of goldfish lymphocytes has not been examined. In the present study, goldfish PBL responded to stimulation with PMA and A23187 *in vitro*. When used alone, PMA induced significantly lower proliferative response while A23187 failed to induce proliferation of the cells. The synergistic effect of PMA and A23187 on goldfish PBL proliferation is similar to that observed for channel catfish PBL [Miller and Clem, 1988]. However, PMA alone induced significant proliferation of lymphocytes in goldfish, but not in channel catfish indicating the species differences in the lymphocyte response to these mitogens.

In fish, as in other higher vertebrates, macrophages participate in both non-specific and specific immune responses. They actively participate in inflammatory responses by migrating towards the site of injury or infection, where chemoattractants are released by invading microorganisms or by activation of complement [Griffin, 1984; MacArthur *et al.*, 1985; stG Howell, 1987]. Macrophages non-specifically engulf foreign particles and invading microorganisms forming the first non-specific defense for infections [Avtalion and Shahrabani, 1975]. They also participate in the specific immune responses by exerting their antigen presenting function.

Fish macrophages have similar morphological and physiological characteristics to mammalian and avian macrophages. Phagocytosis is the best studied function of fish macrophages [Avtalion and Shahrabani, 1975; MacArthur, and Fletcher, 1985; Olivier *et al.*, 1986]. After stimulation, fish macrophages produce several reactive oxygen species such as O_2^- and H_2O_2 with potent microbicidal activities [Chung and Secombes, 1988].

Most of the work on the function of fish macrophages was done in trout and carp using primary macrophage cultures. In my examination of the functions of goldfish macrophages, I established a long term goldfish macrophage cell line. The macrophage cell line has been characterized using the following functional assays: 1) chemotaxis: the cultured macrophages actively migrate towards EAGFS; 2) phagocytosis: the cultured macrophages engulf SRBC, and this phagocytosis is enhanced after opsonization of the SRBC with antiserum to

SRBC; 3) production of reactive oxygen intermediates: the cultured macrophages produce O_2^- upon activation as determined by NBT reduction assay; and 4) production of nitric oxide: the cultured macrophages synthesize and release reactive nitrogen intermediates after appropriate stimulation.

Oriented migration of leukocytes including macrophages resulting in accumulation of immune cells at sites of tissue injury is one of several recognized components of the inflammatory response of mammals. The chemical mediators (chemoattractants) may be of either microbial or host origin [stG Howell, 1987]. Chemotactic movement of leukocytes has been intensively investigated in mammals, primarily in murine and human systems [Snyderman and Goetzl, 1981]. However, this important immune response has been virtually ignored in lower vertebrates, although the chemotaxis of leukocytes has been demonstrated in a few fish species including trout [Griffin, 1984], carp [stG Howell, 1987], plaice [MacArthur *et al.*, 1985], and nurse shark [Obenauf and Hyder Smith, 1985]. The results of the resent study indicate that goldfish macrophages migrated actively towards EAGFS and that the degree of migration was dependent upon the concentration of EAGFS.

Phagocytosis is another important property of fish macrophages. This process functions in both non-specific and specific immune responses. It forms the first line of non-specific defense against invaders. Phagocytosis of macrophages is weak and ineffective in the early stage of non-specific immune responses [Cohn, 1986]. In specific immune responses, the phagocytic ability of macrophages is enhanced by the opsonizing effect of specific antibodies and anaphylatoxins of the activated complement. The activation of macrophages by T lymphocyte products such as interferon γ (IFN γ) also facilitates the process of phagocytosis and degradation of the engulfed particular antigens. Phagocytosis is the first step in antigen processing and presentation [Vallejo *et al.*, 1992] and serves as a trigger for the activation of macrophages [Graham *et al.*, 1988].

Unlike lymphocytes, macrophages do not possess specific receptors for individual antigens, instead, they utilize several groups of "common" receptors (their ligands are shared by more than one species of organisms) to execute their phagocytic activities. Several groups of non-specific receptors have been identified on mammalian macrophages: receptors for the Fc region of immunoglobulins, receptors for activated components of the complement and receptors for carbohydrates [Basu *et al.*, 1991; Kimura and Griffin, 1992; Newman *et al.*, 1991]. Fish macrophages can engulf any type of antigens although they do not have specific

antigen receptors [Secombes *et al.*, 1988]. In addition, the phagocytosis of bacteria by trout macrophages is enhanced by specific antibody [Griffin, 1983; Sakai, 1984] suggesting that trout macrophages may bear Fc receptors on their surface.

Goldfish macrophages actively engulf SRBC, and this process increases significantly when the SRBC are coated with rabbit anti-SRBC serum suggesting that goldfish macrophages, like trout macrophages, may possess Fc receptors for immunoglobulins. Rainbow trout phagocytes showed significantly higher engulfment of the pathogen *Yersinia rukeri* when the bacteria were opsonized with specific antiserum [Griffin, 1983]. Weissmann and colleagues [1978] stated that dogfish (*Mustelus canis*) phagocytes possess surface Fc receptors for heat-aggregated, but not native, immunoglobulins. Studies of antigenic relationships among fish IgM and the high molecular weight immunoglobulins of the other vertebrates, including mammals, indicated that fish μ chains may share epitopes common to μ chains of other craniate taxa. Litman and Marchalonis [1982] argued that the μ chain sequence has been highly conserved, and that IgM arose early in craniate evolution.

After activation with cytokines or mitogens, macrophages characteristically undergo a series of metabolic changes that culminate in the highly active "killer" macrophages. These macrophages cause many changes upon contact with the tumor target cells: inhibition of aconitase, the citric acid cycle enzyme [Drapier and Hibbs, 1986], and inhibition of mitochondrial respiration [Iyengar *et al.*, 1987]. Interruption of these vital metabolic pathways in tumor cells is independent of the respiratory burst and is mediated by L-arginine-derived inorganic nitrogen oxides, notably nitric oxide, produced by activated cytotoxic macrophages [Hibbs *et al.*, 1987; Stuehr and Nathan, 1989]. Nitric oxide is a molecule responsible for the microbicidal and tumoricidal effects of macrophages [Green *et al.*, 1990]. It is also an endothelium-derived relaxing factor [Palmer *et al.*, 1987; 1988]. A number of cells including macrophages [Hibbs *et al.*, 1987], neutrophils [Wright *et al.*, 1989], epithelial cells [Palmer *et al.*, 1988], hepatocytes [Curran *et al.*, 1989] and some tumor cells [Amber *et al.*, 1988] produce nitric oxide. The synthesis of nitric oxide by mammalian cells is dependent upon L-arginine. Two groups of enzymes responsible for nitric oxide generation have been characterized: constitutive and inducible nitric oxide synthase [Stuehr and Griffith, 1992]. In mammalian macrophages, the enzyme responsible for nitric oxide synthesis belongs to the inducible form, which has been cloned recently [Lowenstein *et al.*, 1992; Lyons *et al.*, 1992]. The production of nitric oxide in macrophages begins several (4-6)

hours after cytokine and LPS stimulation and can be quantitatively measured with Griess reaction for nitrite, an oxidative product of nitric oxide, since nitric oxide is unstable.

Using cultured goldfish macrophages, I demonstrated for the first time that fish macrophages synthesize nitric oxide. Goldfish macrophages synthesized maximal amount of nitric oxide after stimulation with 20 μg per ml of LPS for 96 hrs. This is the first report of nitric oxide production not only by fish macrophages but by any fish cell. The ability of fish macrophages to synthesize nitric oxide suggests that fish macrophages share the effector molecules for microbicidal and tumoricidal activities with mammalian macrophages. These findings suggest that fish macrophages may have the same level of evolution as mammalian macrophages, or, on the other hand, mammalian macrophages may not progress beyond the early vertebrate macrophages from the view point of nitric oxide production, which is the major effector molecule for the microbicidal and tumoricidal actions of activated macrophages.

Activation of macrophages by phagocytosis or chemical stimulation triggers the production of reactive oxygen moieties with potent microbicidal activities. This phenomenon is termed the respiratory burst. A number of reactive oxygen metabolites including O_2^- and H_2O_2 are generated after the activation of NADPH oxidase in the membrane of phagocytes. These reactive oxygen intermediates represent the oxygen-dependent killing mechanisms of mammalian phagocytes [Cohen *et al.*, 1980]. Respiratory burst of phagocytes has been intensively studied in mammals and has been demonstrated in several fish species including channel catfish [Scott *et al.*, 1985], striped bass, *Morone saxatilis*, [Stave *et al.*, 1983] and rainbow trout [Chung and Secombes, 1987; Higson and Jones, 1984; Plytycz *et al.*, 1989]. Goldfish macrophages, like rainbow trout phagocytes [Chung and Secombes, 1988], produce O_2^- after stimulation with LPS and PMA as detected by NBT reduction assay. The co-stimulation of the cells with LPS and PMA yielded much higher O_2^- production than the stimulation with LPS or PMA alone indicating synergistic interaction between LPS and PMA.

In conclusion, goldfish lymphocytes and macrophages showed similar functional properties to that of mammalian and other species of fish cells. The assays described in this chapter were used for the assessment of host defense response of goldfish to *T. danilewskyi* following treatment with sex and adrenal steroids.

Table 3-1. The culture medium for goldfish leukocytes.

REAGENTS OR SOLUTIONS	AMOUNT (ml or g)	SPECIAL REQUIREMENT
Milli Q® water	600 ml	Endotoxin-free
Hank's solution	80.0 ml	10 X, no Ca^{2+} & Mg^{2+}
MEM amino acid solution	25.0 ml	50 X
MEM non-essential amino acid solution	25.0 ml	100 X
$NaHCO_3$	2.52 g	
NaOH	0.30 ml	1N
Sodium pyruvate solution	25.0 ml	10 mM
MEM vitamin solution	20.0 ml	100 X
Nucleic acid precursor sol	20.0 ml	2.5 mM*
L-glutamine solution	20.0 ml	200 mM
Gentamicin solution	2.00 ml	50 mg/ml
2-Mercaptoethanol solution	2.00 ml	50 mM, freshly prepared
HEPES	4.00 g	
Insulin	0.01 g	
GFL-15 Medium	1000 ml	

*Nucleic acid precursor solution containing 2.5 mM each of adenosine, cytidine, guanosine, hypoxanthine, thymidine, uridine.

Table 3-2. Phagocytosis by cultured goldfish macrophages at different ratios of macrophage to non-opsonized SRBC. The macrophages (5×10^4 in 200 µl of medium) were incubated with different numbers of SRBC (2.5×10^4 to 4×10^5) at 20°C for 2 hrs in polypropylene tubes. The SRBC that were not engulfed by macrophages were lysed and the macrophages were centrifuged to make slides using a cytocentrifuge and stained using LeukoStat™ Solution. The percent of macrophages that engulfed SRBC was determined by counting 200 macrophages using a microscope. Statistics analysis using Fisher's exact test showed that there are significant differences only between the ratios of 1 : 5 and 1 : 20 ($p = 0.014$), 1 : 5 and 1 : 40 ($p < 0.001$), 10 : 40 ($p = 0.001$) groups. The number of SRBC per macrophage was determined by enumerating the number of SRBC in 50 macrophages. (MΦ = macrophages).

RATIO	PHAGOCYTOSIS	
(MΦ : SRBC)	Percent of Positive MΦ	No. of SRBC per MΦ
1 : 5	10.3	1.07 ± 0.23
1 : 10	14.6	1.65 ± 0.2
1 : 20	24.1	2.27 ± 0.25
1 : 40	35.6	3.23 ± 0.36

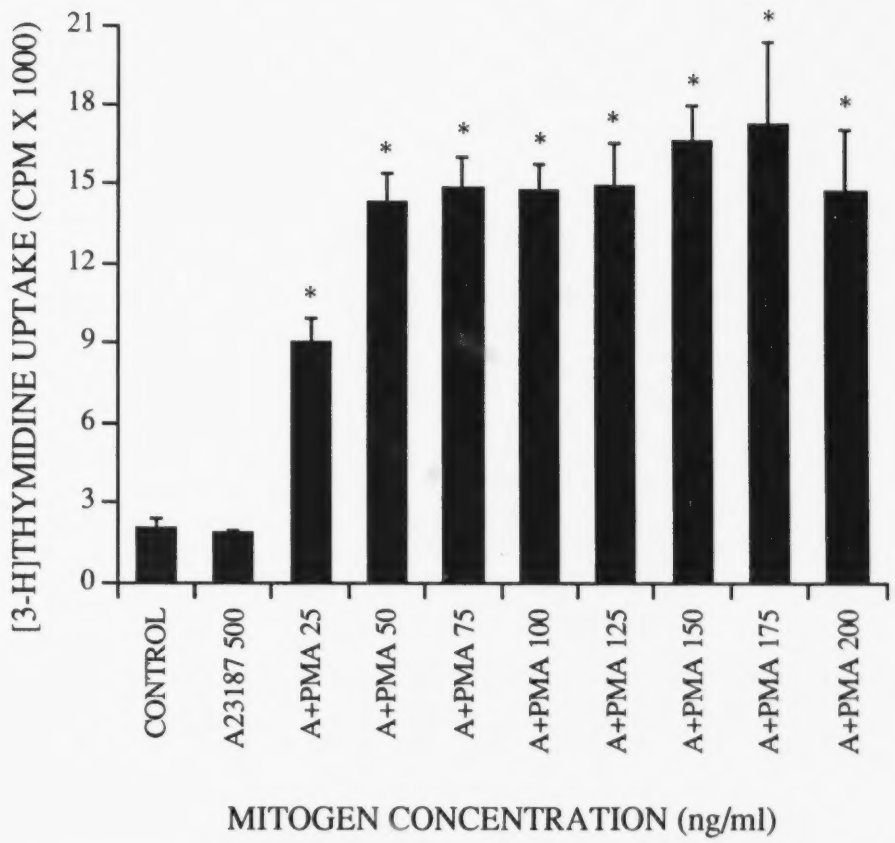


Figure 3-1. PMA dose-response for induction of proliferation of goldfish PBL in the presence of A23187. The cells were treated with A23187 at a concentration of 500 ng per ml and different doses of PMA (25 to 200 ng per ml). Control represents the cells incubated in medium without A23187 and PMA. Each bar represents the mean CPM of [^{3}H]thymidine incorporation of the cells ± standard error of the mean (SEM) of 3 separate experiments (n = 9; there were no significant differences between experiments). Stars indicate the significant differences between control and mitogen-treated groups ($p<0.05$; t-test).

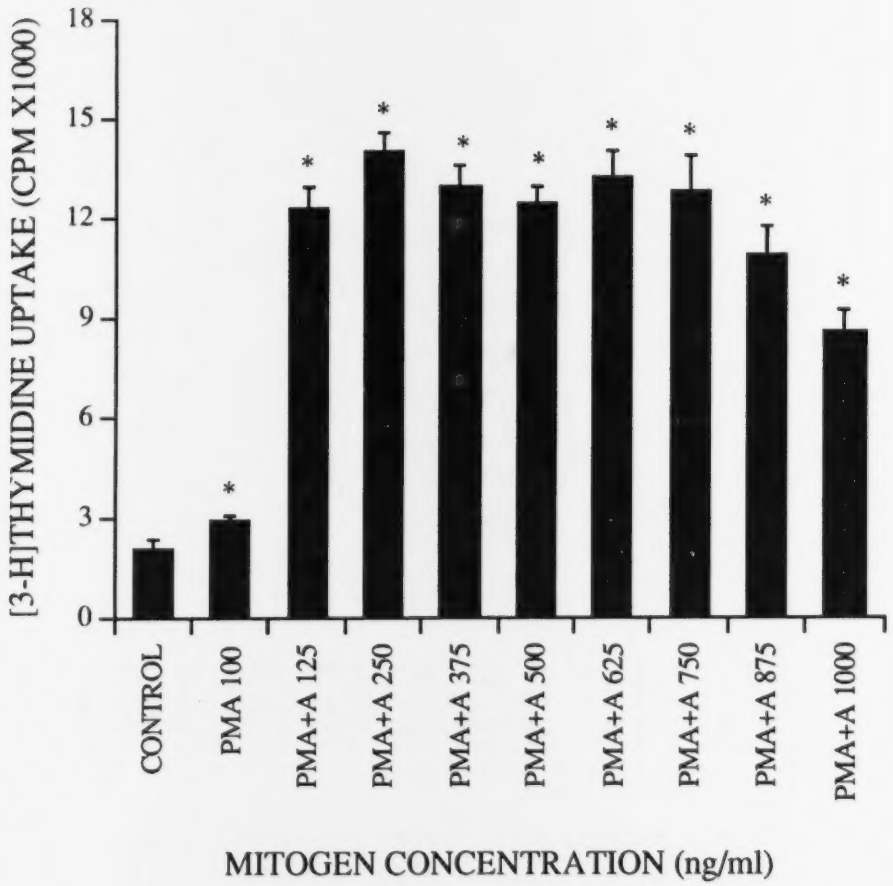


Figure 3-2. Calcium ionophore dose-response for the induction of proliferation of goldfish PBL in the presence of PMA. The cells were treated with constant dose of PMA (100 ng per ml) and different dose of A23187 (125 to 1000 ng per ml). Control represents the cells incubated in medium without PMA and A23187. Each column represents the mean CPM of [^{3}H]thymidine incorporation of the cells ± SEM of 3 separate experiments (n = 9; there were no significant differences between experiments). Stars indicate the significant differences between control and mitogen-treated groups ($p<0.05$; t-test).

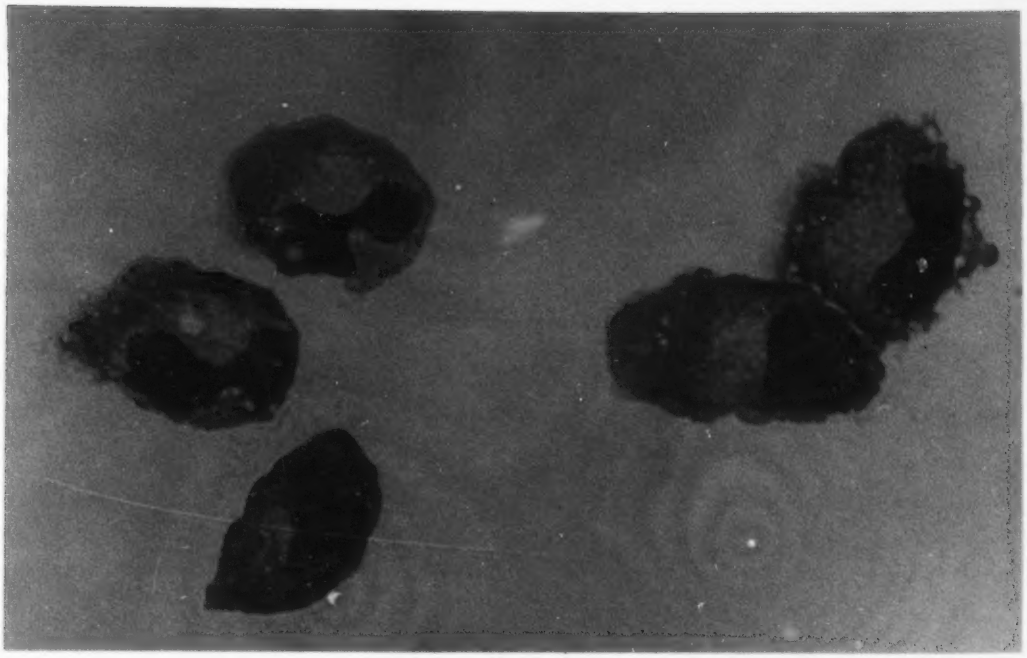


Figure 3-3A. Bright field micrograph of cultured goldfish macrophages (X 1000). The cells of a 18 month-old culture were centrifuged using a cytocentrifuge to make slides and stained with LeukoStat. The photograph was taken using a Nikon MICROPHOT-FXA microscope.

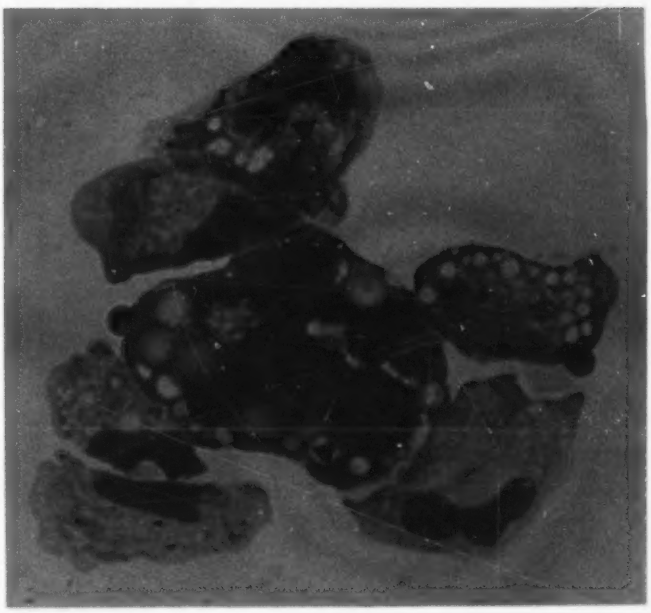
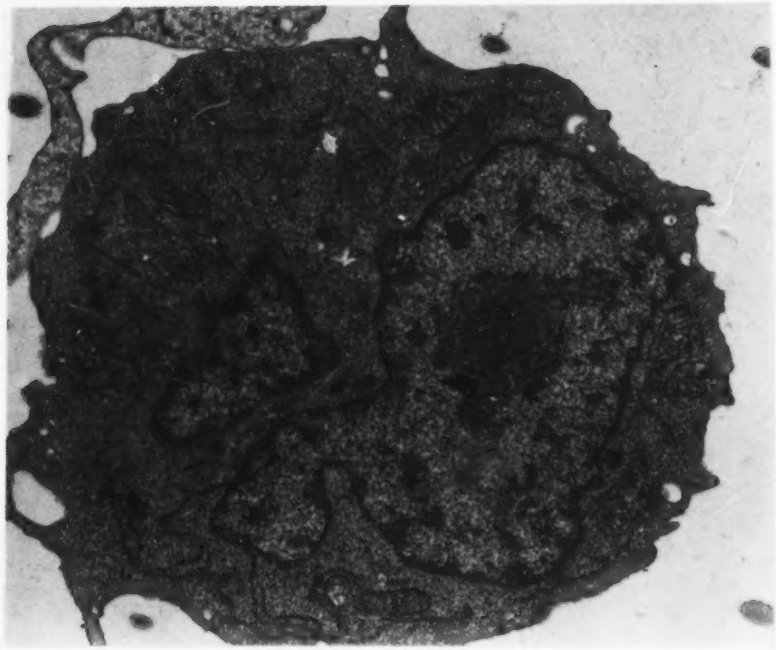


Figure 3-3B: Bright field micrograph of cultured goldfish macrophages with engulfed SRBC (X 1000). The cells of a 18 month-old culture were incubated with SRBC, centrifuged using a cytocentrifuge to make slides, and stained with LeukoStat. The photograph was taken using a Nikon MICROPHOT-FXA microscope. Arrows indicate engulfed SRBC.



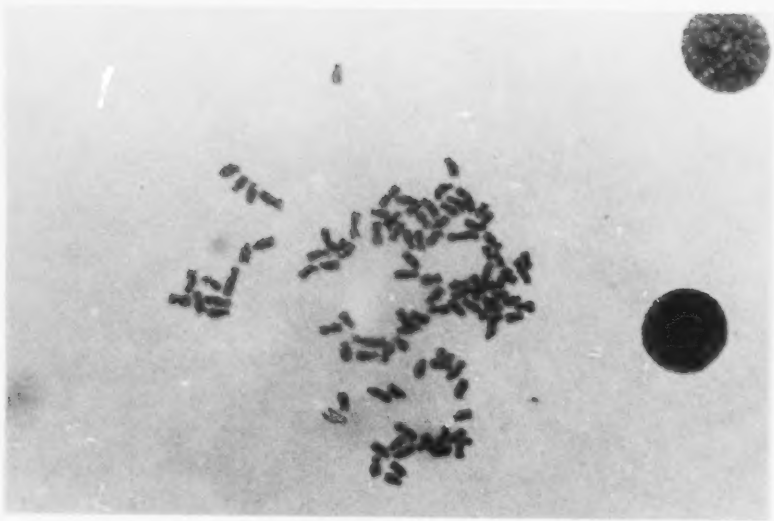


Figure 3-4A. Transmission electron microscope photograph of cultured goldfish macrophages (X 16000).

Figure 3-4B. Karyotype analysis of cultured goldfish macrophages. There are 100 chromosomes in each cell, which is the tetraploid karyotype of goldfish.

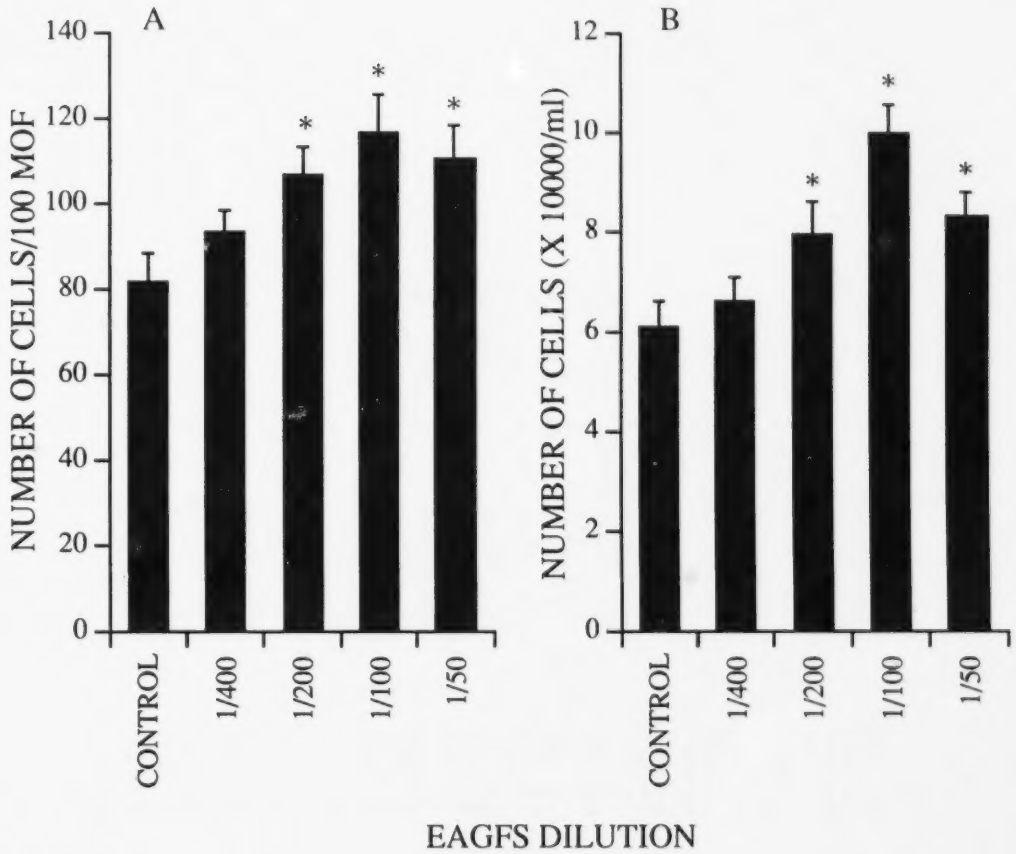


Figure 3-5. Chemotaxis of cultured goldfish macrophages. Endotoxin activated goldfish serum (EAGFS) served as the chemoattractant. Each column in A represents the mean number ± SEM of macrophages that migrated through the pores of a chemotaxis membrane and adhered onto the other side of the membrane and is expressed as the number of cells per 100 microscope oil fields (MOF). Each column in B represents the mean number ± SEM of macrophages that migrated through the pores and entered the medium of the lower chamber and is expressed as the number of cells per ml of medium. The data are from 3 separate experiments (n = 12; there were no significant differences between experiments). Stars indicate statistically significant differences between the control (no chemoattractant) and different EAGFS groups ($p < 0.05$; t-test).

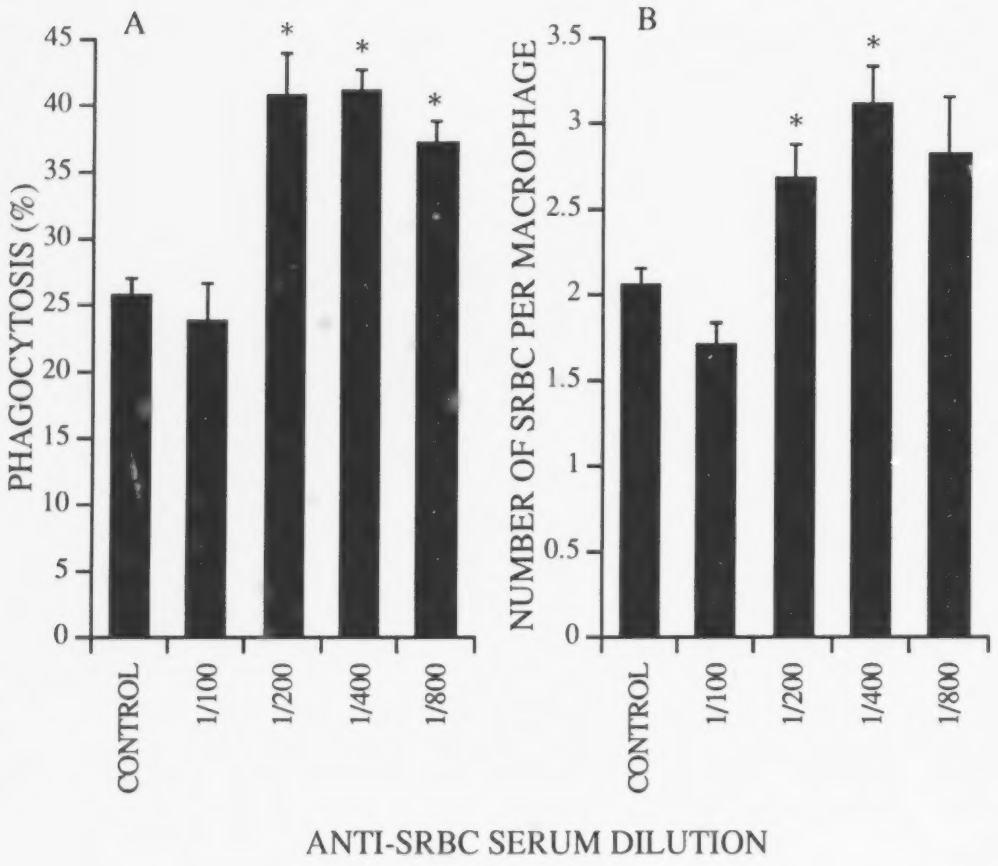


Figure 3-6. Phagocytosis of opsonized SRBC by cultured goldfish macrophages. The ratio of macrophage to SRBC was 1 : 20. Each column in A represents the mean percent of macrophages with engulfed SRBC ± SEM of 2 separate experiments (n = 8). Each column in B represents the mean number of SRBC per macrophage ± SEM of 2 separate experiments (n = 7, there were no significant differences between experiments). Stars indicate statistically significant differences between non-opsonized and opsonized SRBC used as targets ($p<0.05$; t-test).

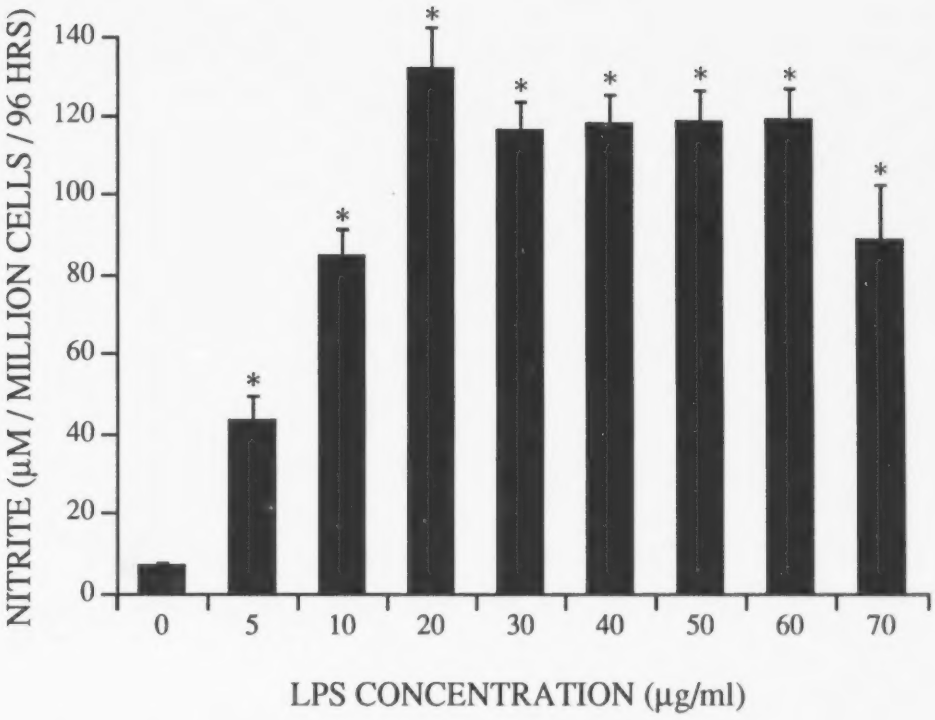


Figure 3-7. Nitrite production of cultured goldfish macrophages stimulated with bacterial LPS. Each bar represents the mean nitrite concentration expressed in µM per 10^6 cells per 96 hrs ± SEM of 3 separate experiments (n = 10; there were no significant differences between experiments). LPS at concentrations tested induced statistically significant increase in nitrite production compared with no LPS control. Stars indicate significant differences between control and different concentrations of LPS ($p<0.05$; t-test).

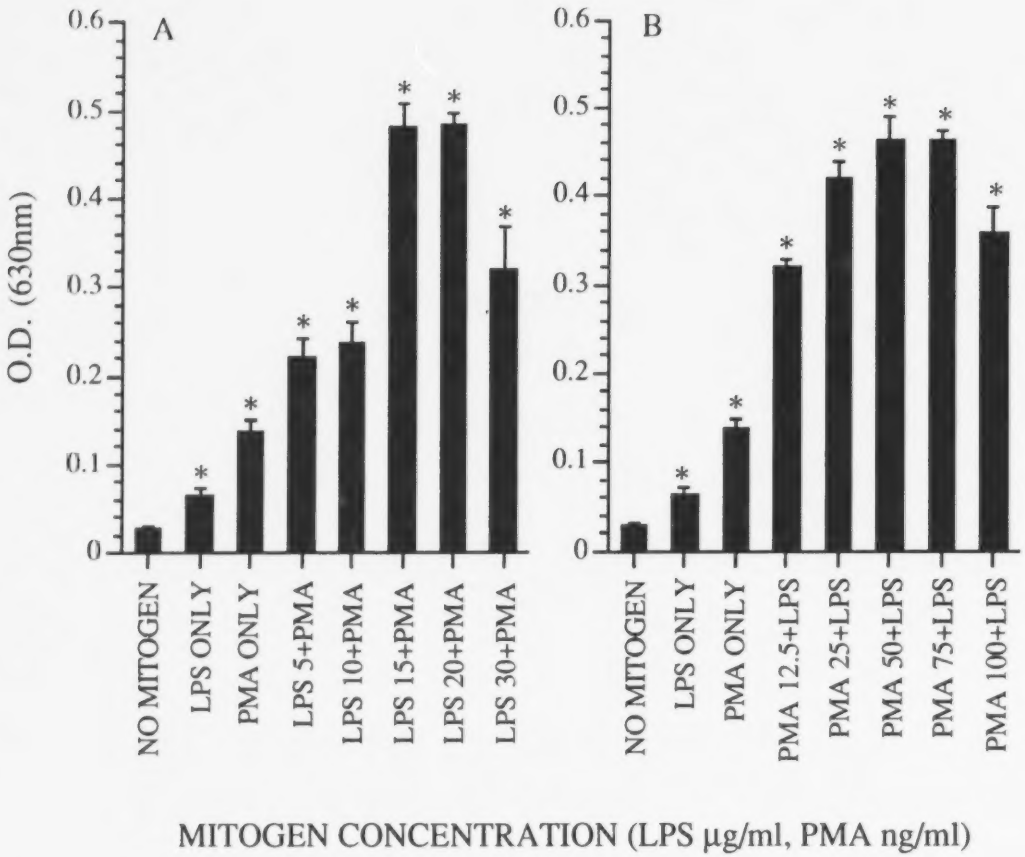


Figure 3-8. Respiratory burst of cultured goldfish macrophages detected by NBT reduction. Each bar represents the mean optical density (O. D.) value ± SEM of a representative of 3 separate experiments (n = 4). LPS concentration was 20 µg per ml for LPS only groups and PMA concentration was 50 ng per ml for PMA only groups. In A, a fixed concentration of PMA at 50 ng per ml and LPS at concentrations of 5 to 30 µg per ml were used. In B, a fixed concentration of LPS at 20 µg per ml and PMA at concentrations of 12.5 to 100 ng per ml were used. All the mitogen-treated groups showed significantly higher O_2^- production when compared to no mitogen controls or LPS only and PMA only groups. Stars indicate the significant differences between control and different concentrations of LPS and PMA combination ($p<0.05$; t-test).

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Chapter Four: The Course of *Trypanosoma danilewskyi* Infection in Goldfish*

INTRODUCTION

The fresh-water fish haemoflagellate, *T. danilewskyi*, has been used in several studies dealing with the biology of the parasite and with regards to the immunological responses mounted by the experimental host the goldfish [Lom, 1973; Woo, 1969; 1981a; 1981b; Woo *et al.*, 1983]. This parasite naturally infects common carp and related fish species in Central and East Europe [Woo, 1981a]. The glossophonoid leech, *Hemiclepsis marginata* is the intermediate host for this flagellate in nature. The development of the infective stage, known as the metacyclic trypanosome or metatrypanosome, occurs in the crop of the leech within 10 days. The parasite is transmitted when the infected leech feeds on a new host [Qadri, 1962].

Goldfish can be successfully infected by intraperitoneal inoculation of trypanosomes and show a dose-dependent response to *T. danilewskyi* infection. High doses of *T. danilewskyi* induce up to 80% mortality in infected goldfish [Lom 1973; Woo 1981b].

The hosts acquire long lasting immunity against *T. danilewskyi* after the initial exposure and elimination of the primary infection [Lom 1973]. The protection for challenge in the surviving fish has been reported to be at least 190 days after the primary infection. Woo [1981b] reported that specific antibodies against the parasite are responsible for this protection, although no attempts were made to examine the cell-mediated immune responses during both the primary and challenge infection.

The purpose of the experiments in this part of my research was to establish and characterize primary and challenge infections with *T. danilewskyi* in goldfish and to assess the host mortality induced by this parasite.

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*Some of the data from this chapter have been published in the *Journal of Fish Diseases* 17:47-56, 1994.

MATERIALS AND METHODS

Fish:

Goldfish were purchased when 4 to 5 cm long from either Ozark Fisheries Inc. or Grassy Forks Fisheries and maintained under the same conditions as described in Chapter 3.

Parasites:

The strain of *T. danilewskyi* used in this study was kindly provided by Dr. P. T. K. Woo, Department of Zoology, University of Guelph, Ontario, Canada. This strain of *T. danilewskyi* was originally isolated by Lom in 1977 from the crucian carp, *C. auratus gibelio*, in Czechoslovakia [Woo, 1981a; 1981b]. The trypanosomes were maintained in our laboratory by intraperitoneal inoculation of goldfish with freshly isolated trypanosomes every 30 days and by *in vitro* cultivation.

Isolation and Purification of *T. danilewskyi* from Goldfish Blood:

Infected goldfish were anesthetized with 0.05% MS222. Whole blood was obtained from the caudal vein using a heparinized syringe (1 ml). The blood was diluted (1:3) in cold (8°C) *T. danilewskyi* culture medium (TDM) [Wang and Belosevic, 1994] containing 40 U per ml of heparin. This cell suspension (3 ml) was then layered on an equal volume of Ficoll-Paque and centrifuged at 350 x *g* for 24 min. at 4°C. The leukocytes and the haemoflagellates at the Ficoll-medium interface were removed with a pipette and transferred to a 15 ml polypropylene centrifuge tube and washed 2 times with cold TDM at 450 x *g* for 10 min. at 4°C. The suspension of parasites and host leukocytes was then placed into 25 cm^2 culture flasks and incubated at 20°C for 1 hr to allow the fish leukocytes to adhere to the plastic. The supernatant containing the haemoflagellates and unattached leukocytes was transferred to new flasks and reincubated at 20°C. The unattached fish leukocytes present in cultures were non-viable after 72 to 96 hrs of incubation at 20°C. The solution containing the parasites was then washed by centrifugation at 350 x *g* for 10 min. to remove dead fish leukocytes and the parasites were re-incubated at 20°C. The purified parasites were enumerated using a haemo-

cytometer and were either used for the initiation of the *in vitro* culture or for infection of goldfish.

Infection of Goldfish and Determination of the Parasitaemia

Desired numbers of trypanosomes suspended in 0.2 ml TDM were injected intraperitoneally into goldfish using a 1 ml syringe fitted with a 25 gauge needle. For determination of the course of infection, individual fish were anesthetized and the blood samples collected from the caudal vein every 5 days. The numbers of parasites in the blood samples were determined until day 65 after inoculation, by counting four aliquots of 1 : 10 to 1 : 100 diluted blood using a haemocytometer. Throughout this thesis the numbers of parasites are expressed per milliliter of blood or as the Log_{10} number of parasites per ml of blood.

Determination of the Packed Red Cell Volume

Packed red cell volume (PRCV) indicates the proportion of erythrocytes in the blood. During the course of infection, including trypanosome infection, the numbers of erythrocytes in the blood decrease resulting in anemia and a correspondent decrease in PRCV. Thus, PRCV is an indicator of the degree of pathogenesis caused by *T. danilewskyi*. To determine PRCV of goldfish, microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) were filled with 0.05-0.1 ml of goldfish blood and centrifuged using a microhematocrit microcentrifuge (model MB IEC, International Equipment Company, Needham Heights, MA) at maximum speed for 5 minutes. The volume occupied by red blood cells (RBCs) was measured with a micro-capillary reader (Damon per IEC Division, Needham, Heights, MA.) and the PRCV was expressed as per cent of the blood volume.

Experimental Design

A. The Course of Infection:

Twenty-four female and 24 male goldfish were divided into 6 groups designated as female and male groups A, B, and C. Eight female or male fish per group were placed in 80 L flow-through aquaria for 3 days of acclimatization before the infection. On the day of infection (day zero), the fish in each group were anesthetized and weighed. Individual fish were marked by fin cutting and were inoculated intraperitoneally with trypanosomes using the following doses:

2.5×10^3 (group A), 2.5×10^4 (group B) and 2.5×10^5 (group C) trypanosomes per fish. Every 5 days (in the morning), the fish in each group were anesthetized and were bled (0.1 ml) from the caudal vein. Fifty microliters of blood was added to 450 µl medium and the number of parasites determined. The parasitaemia was monitored until day 65 after inoculation.

B. Mortality of Infected Goldfish:

To examine the dose related mortality of goldfish infected with *T. danilewskyi*, 48 fish (mixed sex) were randomly divided into three groups and placed in 80 L flow-through aquaria for 3 days before infection. The fish were inoculated with 5×10^5, 1×10^6, and 2×10^6 trypanosomes per fish, respectively. The mortality of the infected fish was recorded every 2 days and the dead fish were examined for the presence of the parasites. The fish were monitored until day 40 after infection, at which point no mortality was recorded for a period of one week and the parasitaemia had declined to low levels in most fish. Another group of 16 uninfected fish were kept under same condition as the experimental groups (control).

C. Challenge Infection:

To examine the resistance of surviving goldfish, 3 groups of goldfish (1, 6, and 12 months after elimination of the primary infection) were inoculated intraperitoneally with 2.5×10^5 trypanosomes per fish. The number of parasites in the blood of challenged fish was determined every 10 days until day 30 after inoculation.

D. Packed Red Cell Volume:

Twenty-four female and 24 male goldfish were divided into 6 groups designated as female and male groups A, B, and C. Eight female or male fish per group were placed in 80 L flow-through aquaria for 3 days of acclimatization before the infection. On the day of infection (day zero), the fish in each group were anesthetized and weighed. Individual fish were marked by fin cutting and the blood samples were collected for initial PRCV. The fish were inoculated intraperitoneally with trypanosomes using the following doses: 2.5×10^3 (group A), 2.5×10^4 (group B) and 2.5×10^5 (group C) trypanosomes per fish. Every 5 days (in the morning), the fish in each group were anesthetized and bled (0.1 ml) from the

caudal vein. The blood was placed into micro-capillary tubes for PRCV analysis. The PRCV was monitored until day 60 after infection.

E. The Control Groups:

For the uninfected controls, 8 females and 8 males were treated the same way as experimental groups. These groups of fish were injected intraperitoneally with 0.2 ml of the incomplete medium used to suspend the parasites. They were anesthetized and bled at the same time when the experimental groups were bled. These fish served as uninfected control for the course of infection and mortality experiments and their blood samples were used to determine the normal PRCV.

RESULTS

The Course of *T. danilewskyi*-Infection in Goldfish Given Different Doses of Parasites:

The course of *T. danilewskyi* infection in goldfish was determined by observing the parasitaemia of goldfish infected with freshly isolated parasites at a dose of 2.5×10^5 trypanosomes per fish. The parasitaemia data were graphically presented in two ways: abundance (Fig. 4-1) and intensity (Fig. 4-2), where abundance is defined as the total number of parasites per ml of blood divided by the total number of fish examined, and intensity is defined as the total number of trypanosomes per ml of blood divided by the number of positive hosts examined. Table 4-1 shows the prevalence of infection in goldfish inoculated with different number of *T. danilewskyi*, which is expressed as a percentage of the number of positive animals divided by the total number examined [Margolis *et al.*, 1982]. The trypanosomes induced a dose-dependent infection in goldfish. Larger inoculation resulted in higher number of infected goldfish, while smaller number of parasites caused lower infectivity (Table 4-1).

The course of *T. danilewskyi*-infection in female goldfish is similar to that in males (Fig. 4-1; Fig. 4-2). The parasitaemia appeared on day 5 after inoculation but the parasite numbers in fish blood were low. Thereafter, the parasitaemia increased rapidly to reach a plateau on day 15 to 20 after infection. The average number of trypanosomes in fish blood was 3.5×10^6 per ml of blood. In some fish the parasitaemia was as high as 5×10^8 per ml of blood, without any apparent

appearance of sickness. The mean number of parasites in the blood decreased gradually after the peak and reached a very low level on day 65 after infection.

When the data were plotted by intensity, a less variable pattern of parasitaemia was obtained because the influence of uninfected (no detectable parasitaemia) fish was eliminated.

The Mortality of Goldfish Infected with *T. danilewskyi*:

In a series of studies, I examined the mortality caused by *T. danilewskyi* in goldfish at doses exceeding 3×10^5 parasites per fish. The percent mortality was 31.3% and 75.0% in fish inoculated with 5×10^5 and 2×10^6 trypanosomes per fish, respectively (Table 4-2). Lower mortality (5-10%) was also observed in fish infected with 2.5×10^5 trypanosomes per fish. The period of the highest mortality occurred during the second and third week after infection, a time when the goldfish harboured the highest numbers of parasites in their blood (Fig. 4-1). Mortality occurred earlier when fish were inoculated with higher numbers of parasites (Fig. 4-3). All dead fish were examined for the presence of *T. danilewskyi* in their blood or body fluids and the parasites were observed in all dead fish.

Challenge Infection of Surviving Goldfish:

Goldfish acquire long lasting protective immunity to *T. danilewskyi* infection after successful elimination of the primary infection [Woo, 1981]. To determine the longevity of the protection, 3 groups of surviving fish were challenged 30, 180, and 365 days after elimination of the primary infection. After challenge, the fish were bled and the blood examined for the parasites on 10-day intervals until day 30 after challenge. The fish in these three groups had no detectable parasitaemia and no mortality at any period after the challenge infection. These results suggested that the surviving fish were protected from *T. danilewskyi* infection and that this protection could last at least 365 days.

Packed Red Cell Volume of Infected Goldfish:

The highest parasitaemia paralleled the significant decline of the PRCV in goldfish blood (Fig. 4-1 and Fig. 4-4). The PRCV of infected goldfish began to decline 5 days after the infection. On day 20 after infection, the mean PRCV decreased significantly to 28 ± 3.8 and 26.6 ± 2.9, for male and female goldfish, respectively (Fig. 4-4). The significant decline in PRCV in this period paralleled

the highest numbers of trypanosomes in the blood. In several heavily infected fish, the PRCV declined from around 37% to 5%, with the parasites taking up 9.5% of the blood volume. The decline in PRCV suggested that *T. danilewskyi* induced anemia in goldfish. After the acute phase of the infection, PRCV of infected goldfish increased and reached a higher volume than the initial volume of the same group after day 35 of infection. These results indicate that *T. danilewskyi* induces a dose-dependent mortality and a corresponding decrease in the PRCV of the infected goldfish that paralleled the period of highest parasite density in the blood of infected hosts.

DISCUSSION

Although they are not the natural host for the parasite, goldfish are highly susceptible to *T. danilewskyi* infection. The haemoflagellates appeared in the peripheral blood of infected fish as early as day 3 after inoculation. Most fish had detectable parasitaemia on day 10 after infection and all fish were positive for the parasite at some points during the infection when inoculated with 2.5×10^5 trypanosomes per fish.

The patterns of both dose- and sex-dependent responses of goldfish to the parasite were observed in the abundance and intensity plots, although a more variable pattern was seen in the abundance plot. The difference between the intensity plot and abundance plot comes from the influence of uninfected fish because the abundance presents the total number of parasites divided by the number of fish examined including those uninfected fish, while the intensity presents the total number of parasites divided by the number of positive fish excluding those uninfected fish. More uninfected fish were observed in mid and low dose groups, thus significant difference between the abundance plot and intensity plot was seen in these two groups.

The course of *T. danilewskyi* infection in this study was longer than that reported by Lom (48 days) [Lom, 1973] and similar to that reported by Woo (70 days) [Woo, 1981b]. The elimination of the parasite has been reported to be due to specific antibodies against the parasite since the parasite loses its infectivity when incubated with immune plasma (containing neutralizing antibodies) and the protective effect of immune plasma can be passively transferred to naive hosts [Woo, 1981b]. The passively transferable protection of goldfish against *T. danilewskyi* by

immune plasma is similar to the immune responses of *T. lewisi* infection in rats, where humoral antibodies are believed to play a major role in protection while phagocytosis plays only a subsidiary role [D'Alesandro, 1970]. The neutralizing effects of the specific antibodies in immune goldfish may be similar to those in the immune sera of other fish. For example, sera from immune mirror carp (*C. carpio* L) and channel catfish can immobilize the free-swimming stages of the ciliate *Ichthyophthirius multifiliis*, and this immobilizing action of the immune serum inhibits the infectivity of the parasite and protects the host [Clark *et al.*, 1987; Hines and Spira, 1974].

The mortality of goldfish was also dependent on the size of the initial inoculum. There was no mortality in the control group (uninfected, bled every 5 days). Thirty-one per cent of the infected goldfish died when given 5 x 10^5 trypanosomes per fish. When given a higher dose of *T. danilewskyi* (2 x 10^6 per fish), greater numbers of fish died (75%). These results are in agreement with those reported by Lom [1973] and Woo [1981b]. Dead fish harbored *T. danilewskyi* confirming the report that goldfish would die from the teaming parasitaemia when inoculated with large number of trypanosomes [Woo, 1981]. However, a few fish harbored lower numbers (about 10^5 per ml of blood) of parasite at death. This suggests that other mechanisms may influence the mortality of the infected fish. One possibility is that *T. danilewskyi* suppresses the immune responses of the host making the infected fish susceptible to other pathogens such as bacteria or viruses.

The immunosuppressive effects of haemoflagellate infections in fish have been demonstrated in rainbow trout infected with *Cryptobia salmositica*. The immune response to SRBC or *Y. ruckeri* is suppressed when rainbow trout is infected with *C. salmositica* [Jones *et al.*, 1986; Wehnert and Woo, 1981]. Both humoral and cell-mediated immunity are suppressed and the mortality of the parasite-infected fish is higher when they are consequently exposed to *Y. ruckeri* than those infected with either pathogen alone [Woo, 1987]. The immunosuppressive effects of *C. salmositica* infection in rainbow trout are mediated by physiological duress including progressive anemia, depressed plasma triiodothyronine (T3), thyroxine (T4), protein and glucose concentration, and lowered liver glycogen content [Laidley *et al.*, 1988]. The immunosuppressive effects of parasitic infection have been reported for other fish species such as winter flounder, *Pseudopleuronectes americanus*, whose inflammatory response and immunoglobulin synthesis are suppressed when infected with *Glugea stephani*, an

intracellular microsporidium of flatfish in North America and Europe [Dykova and Lom, 1978; Laudan *et al.*, 1986; Takvorian and Cali, 1981].

It is known that salmonid fish develop microcytic and hypochromic anemia when they are infected with the haemoflagellate *C. salmositica* [Woo, 1979]. Two antigenic components, which are responsible for the anemia in infected rainbow trout, have been demonstrated in *C. salmositica*. One causes direct lysis of trout RBC (lytic component) and is independent of antibody and complement. The other attaches to RBC, forms immune complexes with specific antibody and activates complement resulting in haemolysis. [Thomas and Woo, 1988; Thomas and Woo, 1989]. The present study demonstrated that goldfish also developed anemia when infected with *T. danilewskyi*. In heavily infected goldfish, PRCV was as low as 5% and the parasite took up to 9.5% of the blood volume indicating that *T. danilewskyi* infection induced severe anemia. This evidence is in agreement with the result reported by Nazrul-Islam and Woo [1991] that *T. danilewskyi* induces anemia in goldfish and the severity of the anemia is associated with high parasitaemia. The cause of *T. danilewskyi*-induced anemia in goldfish is not known. Three possible mechanisms may be involved: 1) *T. danilewskyi* may produce toxic molecules that cause haemolysis or inhibit erythropoiesis in goldfish; 2) *T. danilewskyi* may compete for nutrients or hormones that are responsible for erythropoiesis in goldfish; and 3) immune haemolysis may occur due to non-specific adherence of soluble antigens of *T. danilewskyi* to the red blood cells. The precise mechanisms of the anemia during *T. danilewskyi* infection in goldfish remain to be elucidated.

Protective immunity has been demonstrated in fish infected or immunized with protozoa. In *I. multifiliis* infection, the immobilizing antibody in the blood and mucus, which is thought to bind to the infective free-swimming stage (theront), seems to play a major role in the protection. Hosts that recover from the primary infection are protected for up to 8 months [Hines and Spira, 1974]. The ciliary antigens appear to be important for the protective immunity since vaccination with ciliary protein confers protection [Goven *et al.*, 1981]. In *C. salmositica* infection, protective neutralizing antibody has been demonstrated in rainbow trout and the recovered trout are protected against homologous challenge [Wehnert and Woo, 1981; Jones and Woo, 1986].

In the present study, I demonstrated that goldfish acquire long lasting immunity against *T. danilewskyi* infection. The surviving fish are protected against a homologous challenge for at least 365 days. This prolonged protection may be

the result of non-sterile immunity of goldfish to *T. danilewskyi*. It is possible that small numbers of trypanosomes may persist in some internal organs, which would result in continuous priming of the immune system and hence protection. The prolonged protective immunity of goldfish to *T. danilewskyi* infection may be similar to that found in some mammal-trypanosome host-parasite associations. For example, the recovery of mice from *T. musculi* infection leaves the host resistant to homologous challenge and small numbers of *T. musculi* have been found in the vasa recta of the kidneys [Targett and Veins, 1975; Veins *et al.*, 1972]. The precise mechanisms of the protection of goldfish and the establishment of the end point in the duration of the protection await further investigation.

Table 4-1. Prevalence of infection (# infected/# examined) and sample size for the measurement of number of parasites in the blood of female and male goldfish infected with different doses of *T. danilewskyi*. (P. I. = post infection).

A. FEMALE

Days (P. I)	2.5×10^3/fish # Positive/ # examined	%	2.5×10^4/fish # Positive/ # examined	%	2.5×10^5/fish # Positive/ # examined	%
5	0/8	0	1/8	13	3/8	38
10	0/8	0	5/8	63	7/8	88
15	1/8	13	6/8	75	7/8	88
20	1/7	14	2/7	29	8/8	100
25	1/7	14	3/7	43	7/7	100
30	1/7	14	2/7	29	7/7	100
35	2/7	29	3/7	43	7/7	100
40	1/7	14	3/7	43	7/7	100
45	0/7	0	1/7	14	5/7	71
50	0/7	0	1/7	14	6/7	86
55	1/7	14	2/7	29	4/6	67
60	0/7	0	1/7	14	2/6	33
65	0/7	0	1/7	14	2/6	33

B. MALE.

Days (P. I)	2.5×10^3/fish # Positive/ # examined	%	2.5×10^4/fish # Positive/ # examined	%	2.5×10^5/fish # Positive/ # examined	%
5	0/8	0	0/8	0	3/8	38
10	2/8	25	2/8	25	8/8	100
15	3/8	38	7/8	88	8/8	100
20	2/8	25	7/8	88	8/8	100
25	2/7	29	7/8	88	8/8	100
30	2/7	29	7/8	88	8/8	100
35	2/7	29	7/8	88	7/7	100
40	1/7	14	7/8	88	6/6	100
45	1/7	14	5/8	63	6/6	100
50	1/7	14	5/8	63	5/6	83
55	1/7	14	4/7	57	4/6	67
60	1/7	14	2/7	29	4/6	67
65	0/7	0	2/7	29	3/6	50

Table 4-2. The mortality of goldfish infected with high doses of *T. danilewskyi*. Three groups of 16 fish were inoculated with 5×10^5, 1×10^6, and 2×10^6 trypanosomes per fish, respectively. Number of deaths was recorded every 2 days until day 40 after infection. Statistical analysis using Fisher's exact test showed that there were significant differences between 5×10^5 and 2×10^6 groups ($p = 0.032$), and that there were no significant differences between 5×10^5 and 1×10^6 ($p = 0.716$), or 1×10^6 and 2×10^6 groups ($p = 0.149$). There was no mortality in the uninfected control group.

DOSE	MORTALITY	
(# parasites/fish)	# Dead/# Inoculated	(%)
5×10^5	5/16	31.3
1×10^6	7/16	43.8
2×10^6	12/16	75.0

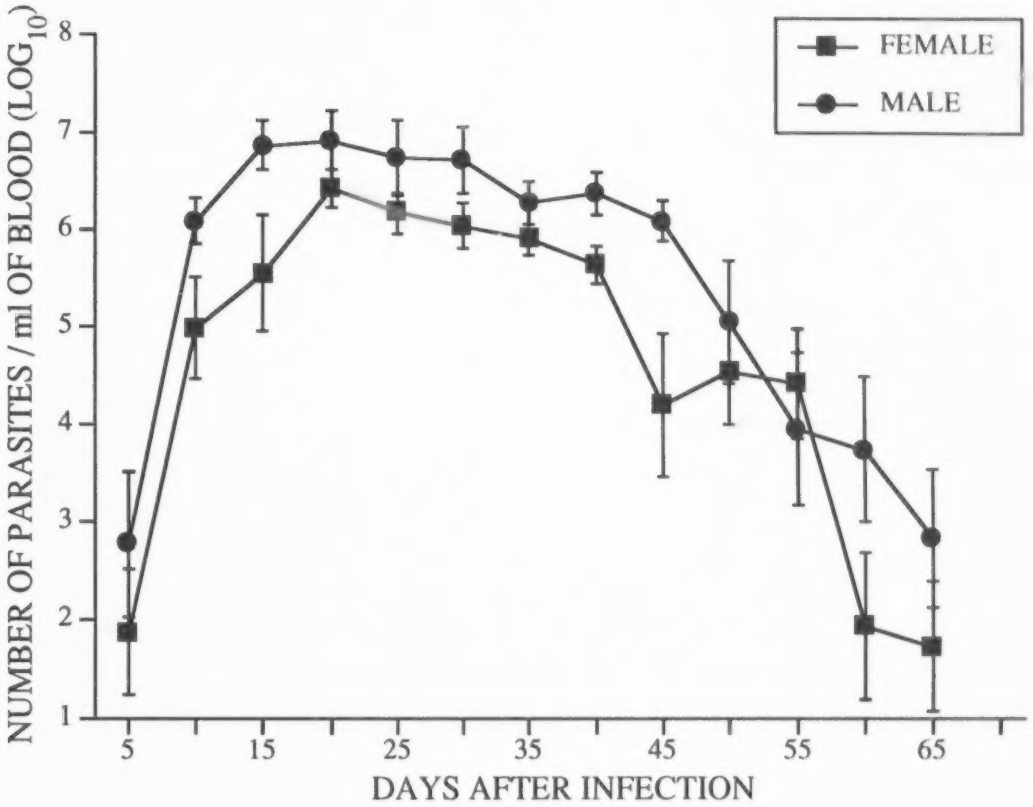

Figure 4-1. The abundance of *T. danilewskyi*-infection in female and male goldfish as measured by parasite numbers in the blood. Abundance is defined as the mean number of parasites for all goldfish examined, including negatives. Female and male goldfish were infected intraperitoneally with 2.5×10^5 parasites per fish. The number of parasites in fish blood was examined every 5 days using a hemacytometer. Each point represents the geometric mean number of parasites per milliliter of blood ± SEM of 8 female or male goldfish.

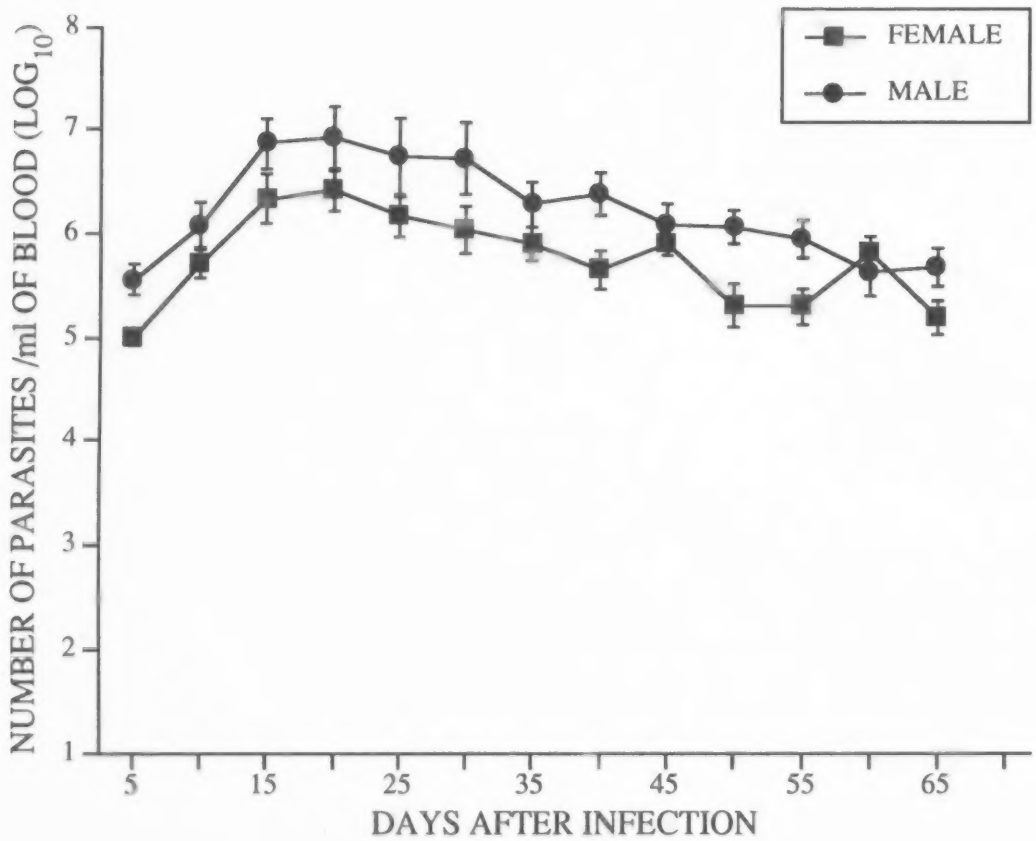

Figure 4-2. The intensity of *T. danilewskyi*-infection in female and male goldfish as measured by number of parasites in the blood. Intensity is defined as the mean number of parasites per positive animal. Female and male goldfish were infected intraperitoneally with 2.5 x 10^5 parasites per fish. The number of parasites in fish blood was examined every 5 days using a hemacytometer. Each point represents the geometric mean number of parasites per milliliter of blood ± SEM of 8 female or male goldfish.

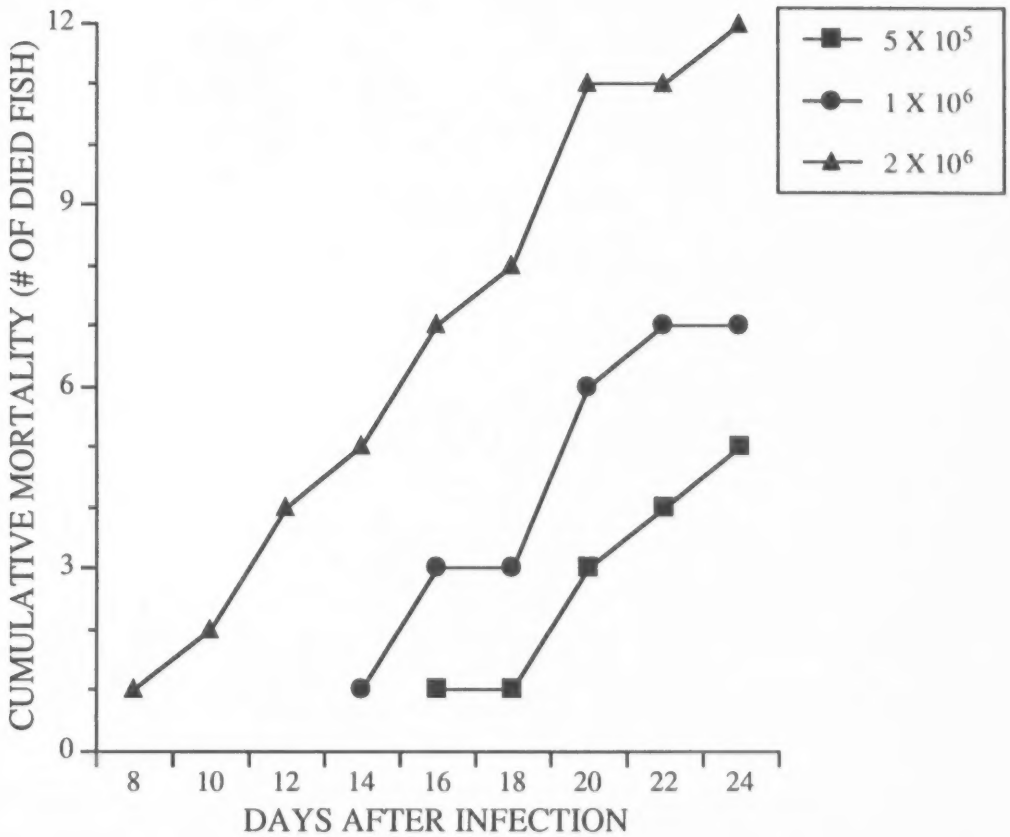

Figure 4-3. The cumulative mortality of goldfish infected with different number of *T. danilewskyi*. Three groups of 16 fish were infected intraperitoneally with 5 x 10^5, 1 x 10^6, and 2 x 10^6 parasites per fish , respectively. The mortality was examined every 2 days by recording the number of dead fish. There was no mortality in uninfected control groups.

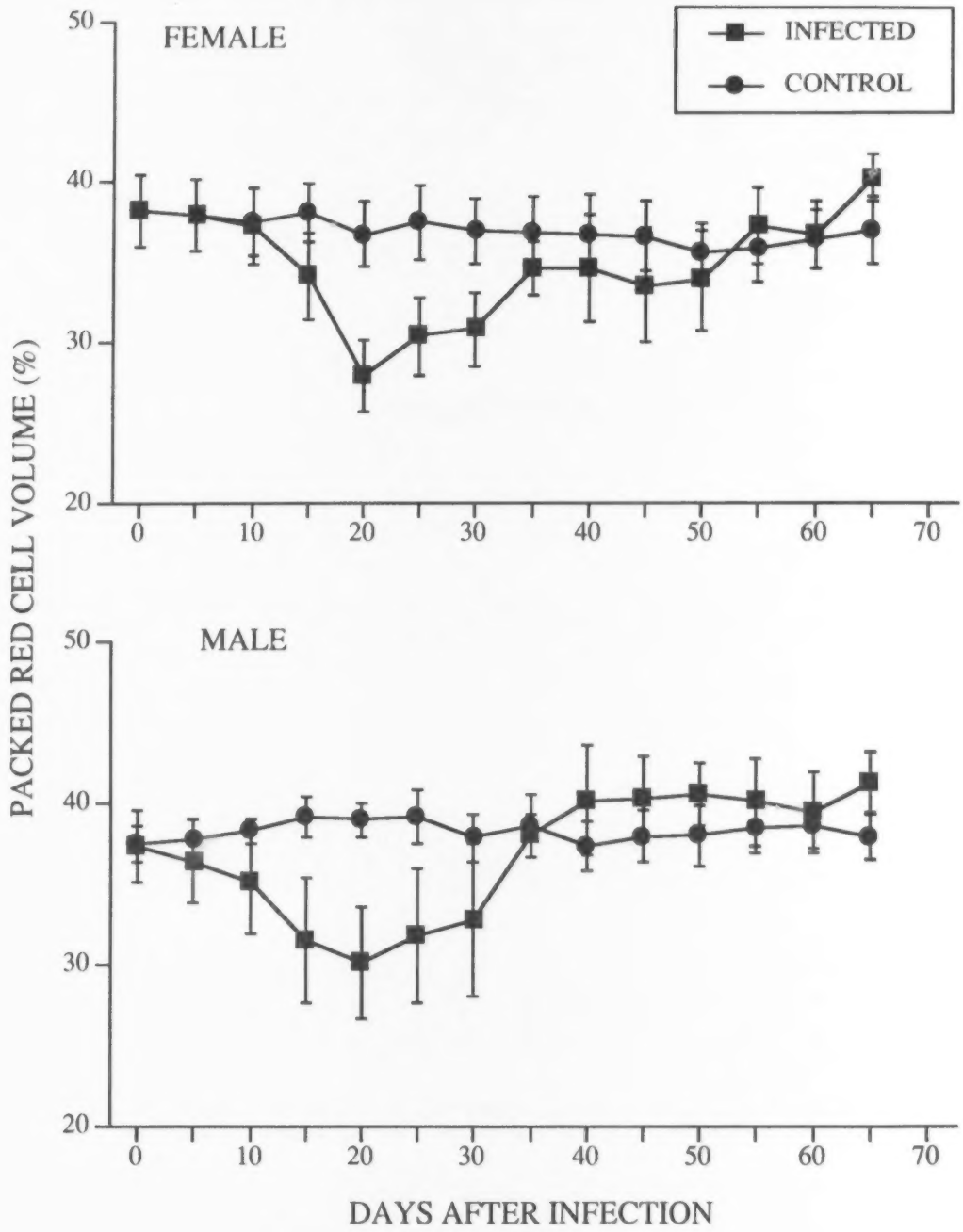

Fig. 4-4. Packed red cell volume in the blood of female and male goldfish infected with 2.5 x 10^5 parasites per fish. Each point represents the percent PRCV (mean ± SEM) of 8 female or 8 male goldfish.

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Chapter Five: The *in vitro* Effects of Estradiol and Cortisol on the Function of Goldfish Lymphocytes and Macrophages

INTRODUCTION

Sex differences in pathophysiological responses have been demonstrated in a number of autoimmune diseases such as lupus erythematosus, rheumatoid arthritis, and many infectious diseases including parasitic protozoan infections in mammals. The sex steroids, including both male and female sex steroids, have been thought to be contributors to this difference [Grossman, 1984; 1985]. The female steroid estradiol inhibits the proliferation of mononuclear leukocytes in mammals [Magnusson and Einarsson, 1990; Magnusson and Fossum, 1992]. Estradiol also down-regulates Class I and Class II major histocompatibility complex antigen expression of guinea pig blood leukocytes [Debout *et al.*, 1991] and influences the function of neutrophils and eosinophils [Josefsson *et al.*, 1992; Lee *et al.*, 1989; Magnusson, 1991] in mammals. However, there is no data concerning the effects of female sex steroids on the functions of leukocytes in fish.

The immunoregulatory property of glucocorticoids has been well defined in animals including mammals and fish. In physiological concentrations, glucocorticoids play an important role in regulation of the metabolism of carbohydrates, lipids and proteins. The concentration of corticoids in the blood of animals increases drastically under stress. Glucocorticoids have been demonstrated to inhibit the function of a variety of mammalian leukocytes including T and B lymphocytes [Bowen and Fauci, 1984; Culpepper and Lee, 1987], NK cells [Gatti *et al.*, 1987; Holbrook *et al.*, 1983; Sulke *et al.*, 1985], macrophages [Lew *et al.*, 1988; Snyder and Unanue, 1982], neutrophils [McCall *et al.*, 1991], eosinophils [Lamas *et al.*, 1991], and basophils [Schleimer *et al.*, 1982; 1989].

The immunosuppressive effects of glucocorticoids have also been reported in fish. For example, glucocorticoids suppress the mitogen-induced lymphocyte proliferation in rainbow trout [Tripp *et al.*, 1987] and plaice [Grimm, 1985]. Cortisone administration depletes the cell populations in the lymphoid organs of rainbow trout and causes histologic lesions in the thymus [Chilmoczyk, 1985]. Cortisol reduces the normal ability of striped bass phagocytes to generate a chemiluminescence response when exposed to bacteria or PMA [Stave and Robertson, 1985]. Cortisol treatment also alters the number and affinity of glucocorticoid receptors in leukocytes and in the gills of coho salmon [Maule and Schreck, 1991].

Lymphocytes and macrophages are the major players in host defense against many parasites including *T. danilewskyi* [Woo and Jones, 1987]. Estradiol is the predominant female sex hormone during spawning in goldfish [Kobayashi *et al.*, 1988] and cortisol is the predominant interrenal steroid in goldfish under stress [Fryer, 1975]. The purpose of this chapter was to assess the influence of the sex steroid, estradiol and the adrenal steroid, cortisol on mitogen-induced lymphocyte proliferation and on macrophage inflammatory function *in vitro*.

MATERIALS AND METHODS

Fish:

Goldfish were purchased from either Ozark Fisheries Inc. or Grassy Forks Fisheries and maintained under the same conditions as described in Chapter 3.

Effects of Estradiol and Cortisol on Mitogen-Induced Proliferation of Goldfish Lymphocytes:

To examine the effects of estradiol and cortisol on the mitogen-induced proliferation of goldfish PBL, goldfish were anesthetized and bled. The lymphocytes were isolated from individual fish using the Ficoll-Paque centrifugation method (see Chapter 3). The isolated cells were seeded into triplicate wells of 96 well microplates (5×10^5 cells per well). To the triplicate wells, appropriate concentrations (0.05, 0.10, 0.50, 1.00 and 5.00 μM) of water-soluble estradiol or cortisol (Sigma, St. Louis, MO) were added. One set of triplicate wells served as untreated control. The plates were incubated at 20°C for 8 hrs. After the incubation, the cells in all wells were stimulated with PMA-A at an optimal concentration (100 ng per ml of PMA and 500 ng per ml of A23187, determined in Chapter 3) for 12 hrs. Fresh medium was added to all the wells after removal of the PMA-A containing medium. The plates were incubated at 20°C for an additional 76 hrs. The cells were treated with 2 μCi per well [^{3}H]thymidine, harvested onto glass microfibre filters using 0.1% SDS in water. The filters were dried in the air, placed into scintillation vials containing 6 ml ScientiVerse™ and counted using a scintillation counter. The experiment was repeated 3 times and the average CPM of the triplicate wells of each experiment was used in the figures and statistics (n = 4).

The Effects of Estradiol and Cortisol on Chemotaxis of Goldfish Macrophages:

To assess the effects of estradiol on the directed migration of goldfish macrophages, two types of experiments were conducted. In the first set of experiments, the cells that migrated through and adhered onto the other side of the Nuclepore membrane were examined. For this purpose, 1 x 10^6 cells were divided into 5 groups (2 x 10^5 cells per group). Four groups of cells were treated respectively with 0.05, 0.10, 1.00, and 5.00 μM estradiol and one group was untreated control. In the second set of experiments, the cells that migrated through the membrane and entered the chemoattractant containing medium were enumerated. For this purpose, 4 x 10^6 cells were divided into 5 groups (8 x 10^5 cells per group). Four groups of cells were treated respectively with 0.05, 0.10, 1.00, and 5.00 μM estradiol and one group was untreated control.

The cells in the estradiol-containing medium were incubated at 20°C for 8 hrs. After the incubation, the cells in each dose group were divided into 4 aliquots and placed in the top wells of the upper chemotaxis chamber. The bottom chamber was prefilled with 200 μl of GFLM containing 1 : 100 dilution of EAGFS (chemoattractant) and was separated from the top chamber by a Nuclepore membrane. The cell-containing chambers were incubated at 20°C for 2 hrs. The cells on the underside of the Nuclepore membrane were stained and enumerated using a microscope. The cells that migrated through and accumulated in the bottom chamber were enumerated using a haemocytometer.

The effects of cortisol on the chemotaxis of goldfish macrophages were assessed by the same procedure to that used for estradiol. The experiment was repeated once.

The Effects of Estradiol and Cortisol on Phagocytosis of Goldfish Macrophages:

To determine the effects of estradiol on the phagocytosis of goldfish macrophages, 1 x 10^6 cells were divided into 5 groups. Four groups of the cells were incubated with estradiol at concentrations of 0.1, 1.0, 5.0, and 10.0 μM respectively and 1 group of cells was not treated serving as control. These 5 groups of cells were incubated at 20°C for 8 hrs before the phagocytosis assay.

Sheep red blood cells (2 x 10^7) were treated with rabbit anti-SRBC antibodies at 1:200 dilution and incubated at 20°C for 30 min. The antibody-coated SRBC were divided equally into 5 groups, which were mixed with the 5 groups of

macrophages. The cell mixtures in each group were divided into 3 aliquots and incubated at 20°C for 2 hrs. The number of macrophages with engulfed SRBC and the number of SRBC per macrophage were determined using the procedures described in Chapter 3.

The effects of cortisol on phagocytosis of goldfish macrophages were assessed using the same procedure that was employed for estradiol. The experiment was repeated once.

The Effects of Estradiol and Cortisol on Nitric Oxide Production of Goldfish Macrophages:

To determine the effects of estradiol and cortisol on the nitric oxide production of goldfish macrophages, the cells in triplicate wells (5×10^5 cells per well) were treated with estradiol or cortisol at concentrations of 0, 0.1, 1.0, 5.0, 10.0 μM in 200 μl medium per well. After the addition of the steroids, the cells were incubated at 20°C for 8 hrs, and then stimulated with LPS at concentration of 20 μg per ml for 96 hrs. The nitric oxide production was assessed using the procedures described in Chapter 3. The experiment was repeated 2 times.

Effects of Estradiol and Cortisol on Respiratory Burst of Goldfish Macrophages:

To determine the effects of estradiol and cortisol on the respiratory burst of goldfish macrophages, the cells in triplicate wells (1×10^5 cells per well) were treated with estradiol or cortisol at concentrations of 0, 0.1, 1.0, 5.0, 10.0 μM at 20°C for 8 hrs. The hormone treated cells were stimulated with LPS at concentration of 20 μg per ml for 12 hrs at 20°C. The cells were triggered for respiratory burst with PMA (50 mg per ml). The reduction of NBT was assessed using the procedure described in Chapter 3. The data were from a single experiment.

RESULTS

Effects of Steroids on Mitogen-Induced Proliferation of Lymphocytes:

The effects of estradiol and cortisol on the mitogen-induced proliferation of goldfish PBL were examined using the newly established [^{3}H]thymidine incorporation assay. Goldfish PBL were treated with different concentrations of estradiol

or cortisol prior to stimulation with the mitogens PMA+A. The proliferative response of the steroid-treated PBL was examined and compared to the untreated control. Both estradiol and cortisol inhibited the proliferation of goldfish PBL (Fig. 5-1A; Fig. 5-1B). Estradiol, at a concentration of 10^{-6} M, caused a statistically significant decrease ($p = 0.033$) in the [^{3}H]thymidine incorporation, while cortisol, at a concentration of 10^{-7} M, induced a significant decrease ($p = 0.027$) in lymphocyte proliferation.

Effects of Estradiol and Cortisol on Chemotaxis of Macrophages:

To determine the effects of steroids on the chemotaxis of goldfish macrophages, the cells were treated with different concentrations of estradiol or cortisol at 20°C for 8 hrs.

Estradiol at a concentration of 5×10^{-7} M caused a statistically significant decrease ($p = 0.008$) in the number of cells per 100 MOF (Fig. 5-2A) and at 10^{-7} M induced a statistically significant decrease ($p = 0.018$) in the number of cells that migrated through the filter and entered the bottom well of the chemotaxis chamber when compared with the untreated controls (Fig. 5-3A).

Cortisol at a concentration of 10^{-7} M induced statistically significant decrease in the number of cells on the membrane ($p = 0.009$) (Fig. 5-2B). At the concentration of 10^{-7} M, cortisol also induced statistically significant decrease in the number of cells that migrated through the filter and entered the bottom well of the chemotaxis chambers ($p = 0.001$) when compared with the controls (Fig. 5-3B). At higher concentrations, both cortisol and estradiol caused greater inhibition of chemotaxis, indicating that this inhibition is dose-dependent (Fig. 5-2; Fig. 5-3).

Effects of Estradiol and Cortisol on Phagocytosis of Goldfish Macrophages:

To determine the influence of estradiol and cortisol on phagocytosis of goldfish macrophages, the cells were pre-treated with different concentrations of estradiol or cortisol for 8 hrs prior to the addition of opsonized SRBC.

Estradiol at a concentration of 10^{-6} M significantly inhibited the phagocytosis of opsonized SRBC by goldfish macrophages when compared to the untreated controls (Fig. 5-4A). The percent of macrophages that engulfed SRBC decreased by 15% ($p = 0.024$) after estradiol treatment. However, at a concentration of 10^{-7} M estradiol only induced a 6% decrease in phagocytosis of the cells ($p = 0.286$).

At a concentration of 10^{-6} M, cortisol induced a significant decrease in the phagocytosis of the cells when compared to the untreated control (Fig. 5-4B). The percent of macrophages that engulfed SRBC decreased by 20% ($p = 0.013$). At a concentration of 10^{-7} M cortisol only induced an 8% decrease in phagocytosis ($p = 0.125$). At higher concentrations, both estradiol and cortisol caused a further decrease in phagocytosis of SRBC by goldfish macrophages (Fig. 5-4A; Fig. 5-4B).

Estradiol at 10^{-6} M also induced a statistically significant decrease in the number of SRBC per macrophage when compared to the untreated control (Fig. 5-5A). The number of SRBC per macrophage was 2.4 ± 0.16 in the control and decreased to 1.8 ± 0.1 in estradiol-treated macrophages ($p = 0.033$). At a concentration of 10^{-7} M estradiol did not affect the number of SRBC per macrophage ($p = 0.225$). At a concentration of 5×10^{-6} M, cortisol caused a statistically significant decrease in the number of SRBC per macrophage (Fig. 5-5B). The average number of SRBC per macrophage was 2.45 ± 0.11 in control and declined to 1.6 ± 0.1 in treated macrophages ($p = 0.007$). However, cortisol at concentrations lower than 10^{-6} M did not affect the number of SRBC per macrophage (Fig. 5-5B).

Effects of Estradiol and Cortisol on Nitric Oxide Production of Macrophages:

To test the effects of the steroids on nitric oxide production, the macrophages were pre-incubated with different concentrations of estradiol and cortisol at 20°C for 8 hrs and then stimulated with 20 μg per ml of LPS.

Unlike the effects on the chemotaxis and phagocytosis, where estradiol and cortisol exhibited similar but dose-dependent suppressive effects, the effects of estradiol and cortisol on the nitric oxide production of goldfish macrophages were completely different (Fig. 5-6A; Fig. 5-6B). Cortisol, on the other hand, strongly inhibited the nitric oxide production of cultured macrophages. Even at a concentration of 10^{-7} M, cortisol significantly inhibited the nitric oxide production when compared to the untreated control group (Fig. 5-6B; $p = 0.004$).

Effects of Estradiol and Cortisol on O_2^- Production of Macrophages:

Superoxide anion, one of the major products in the process of macrophage activation responsible for the microbicidal effects of the cells, can be quantitatively determined by NBT reduction assay. To test the effects of estradiol and

cortisol on O_2^- production, the macrophages were pre-incubated with estradiol or cortisol at concentrations of 0.1 to 10 μM at 20°C for 8 hrs and then stimulated with 20 μg per ml LPS. The macrophage monolayer was covered with 100 μl of 1 mg per ml of NBT in culture medium containing 50 ng per ml of PMA. The reduction of NBT was quantitatively determined using the procedures described in Chapter 3.

The results showed that both estradiol and cortisol at concentrations of 0.1 to 10 μM had no effects on the O_2^- production of goldfish macrophages (Fig. 5-7A; Fig. 5-7B) suggesting that estradiol and cortisol do not influence the respiratory burst of goldfish macrophages.

DISCUSSION

This study was done to examine the *in vitro* effects of estradiol and cortisol on the proliferation of lymphocytes and the inflammatory function of macrophages in goldfish. In mammals, the mitogen-induced proliferation is the most commonly used assay for evaluation of lymphocyte function. The mitogen-induced proliferative assay for lymphocytes has been reported in several fish species including channel catfish [Miller and Clem, 1988], trout [Kaattari and Yui, 1987], carp [Caspi *et al.*, 1984], and shark [Grogan and Lund, 1990]. In goldfish, the peripheral blood lymphocytes exhibited strong proliferation when stimulated with PMA and ionophore A23187, although there was a considerable difference in the response of lymphocytes between individual fish (see Chapter 3).

The physiological concentrations of cortisol and estradiol in goldfish serum are 10^{-8} to 5 x 10^{-8} M [Fryer, 1975] and 10^{-9} to 10^{-8} M [Kobayashi *et al.*, 1987; 1988], respectively. The concentrations of the steroids that influence the lymphocyte proliferation *in vitro* are 10 to 100-fold higher than the physiological concentrations of the steroids in goldfish serum.

Estradiol is the most important female sex steroid in comparison to other estrogens in goldfish shortly before and during spawning [Kobayashi *et al.*, 1987; 1988] consequently the regulatory effects of estradiol on lymphocyte proliferation may be important in the natural ability of fish to deal with infectious diseases including *T. danilewskyi* infection. The results of the present study show that estradiol at a concentration of 10^{-6} M significantly inhibited the proliferation of goldfish lymphocytes. Cortisol, the predominant interrenal steroid in goldfish

under stress, was used as a positive control because its immunosuppressive effects on lymphocyte proliferation are well documented in vertebrates including bony fish [Grimm, 1985; Stave and Robertson, 1985; Tripp *et al.*, 1987]. At a concentration of 10^{-7} M, cortisol significantly inhibited lymphocyte proliferation.

The inhibiting concentration of cortisol for lymphocyte proliferation in goldfish was similar to the cortisol concentration in the blood of goldfish under stress [Fryer, 1975]. However, the concentration of estradiol that inhibited lymphocyte proliferation *in vitro* was much higher than the physiological concentration of estradiol in the blood of goldfish during spawning [Kobayashi *et al.*, 1987; 1988]. One possible explanation for this is that the serum proteins (sex steroid binding protein) present in the culture medium may have combined with the estradiol, causing formation of protein-estradiol complexes, which is the inactive form of estradiol [Martin, 1980]. A protein, known as sex steroid binding protein, in the blood serves as a transporter carrying estradiol to remote locations. Upon binding to the protein, estradiol loses its ability to pass cell membrane thus loses its bioactivity. The sex steroid binding protein also serves as a "buffer" for estradiol since the binding and releasing are concentration dependent [Petra, 1991]. Very high concentrations of estradiol may be found adjacent to the steroid-synthesizing cells in the gonad where lymphocytes and other leukocytes may encounter the extremely high concentration of estradiol before the combination of estradiol with sex steroid binding protein.

An alternative explanation is that the number and affinity of estradiol receptors in fish leukocytes are lower than traditional estradiol target cells, thus higher concentrations of estradiol are needed to modulate the leukocyte function. It is speculated that steroids execute their regulatory function through a group of intracellular receptors belonging to the steroid hormone receptor superfamily in their target cells. Upon binding to steroids, the receptors interact with specific hormone response elements located in the promoters of numerous genes resulting in the induction of gene expression that control hormone-induced process such as differentiation, cell growth, and homeostasis [Reichel and Jacob, 1993]. The number and affinity of estradiol receptors are regulated by several factors including the presence of the steroids, physiological condition of the cells and alteration of the micro-environment of the cells. It is possible that the number and affinity of estradiol receptors in goldfish leukocytes are down-regulated during the isolation and cultivation process.

Fish macrophages have similar morphological and physiological characteristics to mammalian and avian macrophages. Chemotaxis has been demonstrated in several fish species [Griffin, 1984; MacArthur *et al.*, 1985; stG Howell, 1987]. Phagocytosis is the best studied function of fish macrophages [Avtalion and Shahrabani, 1975; MacArthur and Fletcher, 1985; Olivier *et al.*, 1986]. After stimulation, fish macrophages produce several reactive oxygen intermediates (O_2^- and H_2O_2) with microbicidal activities [Chung and Secombes, 1988]. However, most of the work on fish macrophages was done using primary cultures of macrophages for channel catfish, trout and carp. In the present study I established a goldfish macrophage cell line, which has been maintained in culture for more than 2 years. The cultured macrophages have been characterized by ascertaining that: 1) they actively migrate towards a chemoattractant; 2) they engulf both opsonized and non-opsonized targets; and 3) they produce reactive oxygen and nitrogen intermediates after appropriate stimulation. The development of the macrophage cell line will greatly facilitate the study the role of macrophages in fish defense against pathogens.

Directed migration towards chemoattractants is an important inflammatory function of macrophages. It increases the accumulation of macrophages at the infection site. The anti-inflammatory properties of estrogen has been demonstrated in mammals. Estradiol administration inhibits leukocyte production in bone marrow and affects the distribution of polymorphonuclear cells in the peripheral blood indicating an anti-inflammatory property [Josefsson *et al.*, 1991]. Estradiol also induces a significant decrease in the number of eosinophils in spleen red pulp indicating that this steroid may have regulatory effects on leukocyte migration [Neumann *et al.*, 1987]. In the present study, it has been demonstrated that estradiol at a concentration of 5×10^{-7} M significantly inhibited the migration of goldfish macrophages. The immunological significance of the inhibitory effects of estradiol is that the sex steroids may reduce the accumulation of macrophages at inflammatory sites.

Glucocorticoids are well-known for their anti-inflammatory properties and have been used to treat inflammatory and allergic disorders for many years. Cortisol can both enhance mobilization and inhibit chemotaxis of neutrophils in humans [Davis *et al.*, 1991]. Glucocorticoids can also inhibit eosinophil responses to granulocyte-macrophage colony-stimulating factor and inhibit cytokine-mediated eosinophil survival in humans [Lamas *et al.*, 1991; Wallen *et al.*, 1991]. Dexamethasone inhibits the chemotactic activity of inflammatory leukocytes in the

rat [Kurihara *et al.*, 1984]. In the present study, cortisol at 1 x 10^{-7} M significantly inhibited chemotactic migration of goldfish macrophages, suggesting that cortisol may have anti-inflammatory effects in fish.

An important component of fish host defense is phagocytosis. In mammalian systems, estradiol has been shown to inhibit the phagocytosis of porcine peripheral blood neutrophils [Magnusson, 1991]. Estradiol reduces the responsiveness of human neutrophils as assessed by O_2^- generation and degranulation [Buyon *et al.*, 1984]. Hydrocortisone and dexamethasone have been reported to inhibit the phagocytosis of bone marrow-derived mononuclear cells of mice *in vitro* [van-der-Meer *et al.*, 1986], and dexamethasone has been reported to inhibit the phagocytosis of rat macrophages *in vivo* and *in vitro*. This inhibition may be mediated through changes in macrophage metabolism since the glucocorticoids are known to inhibit the activities of hexokinase, glucose-6-phosphate dehydrogenase, glutaminase and citrate synthase, which are key enzymes of glycolysis, glutamilysis, the pentose-phosphate pathway and the Krebs cycle [Costa-Rosa *et al.*, 1992].

In the present study, incubation of goldfish macrophages with 10^{-6} M estradiol or cortisol resulted in significant decrease in phagocytosis. Both phagocytosis and the number of targets per macrophage significantly decreased after treatment with the steroids. The inhibitory effects of estradiol and cortisol on phagocytosis of goldfish macrophages were dose-dependent. Higher concentrations of estradiol and cortisol caused further decrease in the percent of macrophages with engulfed SRBC and the number of SRBC per macrophage. The inhibition of phagocytosis by the steroids may affect the antigen processing and presenting function of fish macrophages.

Nitric oxide, which was initially described as an endothelium-derived relaxing factor [Palmer *et al.*, 1987], has been demonstrated to be a molecule responsible for the microbicidal and tumoricidal effects of macrophages and neutrophils in mammals [Green *et al.*, 1990]. Macrophages are the major cell type to generate nitric oxide [Hibbs *et al.*, 1987], although other cells including neutrophils [Wright *et al.*, 1989], epithelial cells [Palmer *et al.*, 1988], hepatocytes [Curran *et al.*, 1989] and some tumor cells [Amber *et al.*, 1988] also possess NOS. The synthesis of nitric oxide by mammalian cells is L-arginine-dependent, in which 2 groups of enzymes responsible for nitric oxide generation have been characterized: constitutive and inducible nitric oxide synthases [Stuehr and Griffith, 1992]. Macrophages in mice and rats possess the inducible nitric oxide synthase, which has been cloned recently [Lyons *et al.*, 1992; Lowenstein *et al.*,

1992]. The production of nitric oxide in macrophages begins several hours after cytokine and LPS stimulation and can be quantitatively measured with Griess reaction for nitrite.

In the present study, goldfish macrophages were found to produce nitric oxide in response to LPS stimulation. The capability of fish macrophages to synthesize nitric oxide indicates that the inducible nitric oxide synthase is also expressed in vertebrates other than mammals.

Estradiol and cortisol showed different effects on nitric oxide production of goldfish macrophages. Estradiol had no effect on nitric oxide production of goldfish macrophages. Cortisol, on the other hand, at concentration of 10^{-7} M induced significant inhibition in nitric oxide production. At higher concentrations, cortisol almost completely blocked the nitric oxide synthesis. This difference in modulation of nitric oxide production of macrophages may suggest that the gene products induced by estradiol are different from those induced by cortisol and that they have different effects on the inducible nitric oxide synthase in goldfish macrophages.

These results are in agreement with the findings reported by Pinto and colleagues [1993] who demonstrated that dexamethasone inhibits nitric oxide production by activated J774 macrophages (a mouse cell line). They suggest that the protective effects of glucocorticoids in endotoxin-induced hypotension and their immunosuppressive action may partially depend on the inhibition of the inducible NOS by these steroids. Dexamethasone also inhibits the inducible nitric oxide synthase in rat peritoneal neutrophils and this inhibition may contribute to the anti-inflammatory activity of this and other glucocorticoids [McCall *et al.*, 1991]. This strong inhibition of nitric oxide production of goldfish macrophages by cortisol may contribute to the immunosuppressive action of cortisol in fish under stress since stress can increase cortisol concentration in the blood [Fryer, 1975]. The differences between the inhibitory effects of estradiol and cortisol on nitric oxide production of goldfish macrophages partially reflect the differences of the immunosuppressive actions of the steroids, i.e. cortisol has stronger suppressive effect on immune responses than estradiol since estrogen stimulates antibody responses but inhibits T cell-mediated inflammation [Josefsson *et al.*, 1992].

Membrane stimulation such as adherence of a particle to macrophage membrane or contact with soluble activating agent PMA triggers the respiratory burst. Goldfish macrophages, like trout phagocytes [Chung and Secombes, 1988],

produce O_2^- after stimulation with LPS and PMA as detected by NBT reduction assay. Little is known about the effects of steroids on the respiratory burst of macrophages and neutrophils. Contradictory results have been reported in human neutrophils *in vitro* and mouse neutrophils *in vivo*. Estradiol at pharmacological levels (10^{-5} M) induces small but significant inhibition of O_2^- generation in human neutrophils [Buyon *et al.*, 1984]. Estradiol administration significantly enhances the susceptibility of mice to the disseminated gonococcal infection, and inhibits the bactericidal activity of polymorphonuclear leukocytes mediated by myeloperoxidase, but does not influence the capacity of polymorphonuclear leukocytes to release O_2^- [Kita *et al.*, 1985]. In the present study, the effects of estradiol and cortisol on the respiratory burst of goldfish macrophages were examined. Both estradiol and cortisol failed to inhibit the respiratory burst of cultured macrophages.

The sex steroid estradiol, like cortisol, has suppressive effects on the function of goldfish lymphocytes and macrophages. Estradiol and cortisol inhibited the mitogen-induced proliferation. Both had inhibitory effects on the chemotaxis and phagocytosis of goldfish macrophages, while only cortisol suppressed nitric oxide production by goldfish macrophages. Estradiol and cortisol had no effects on the respiratory burst of the macrophages. The immunosuppressive effects of estradiol and cortisol could influence the capability of fish to defend against infectious diseases such as *T. danilewskyi* during spawning when estradiol and cortisol appear in higher concentrations in the blood.

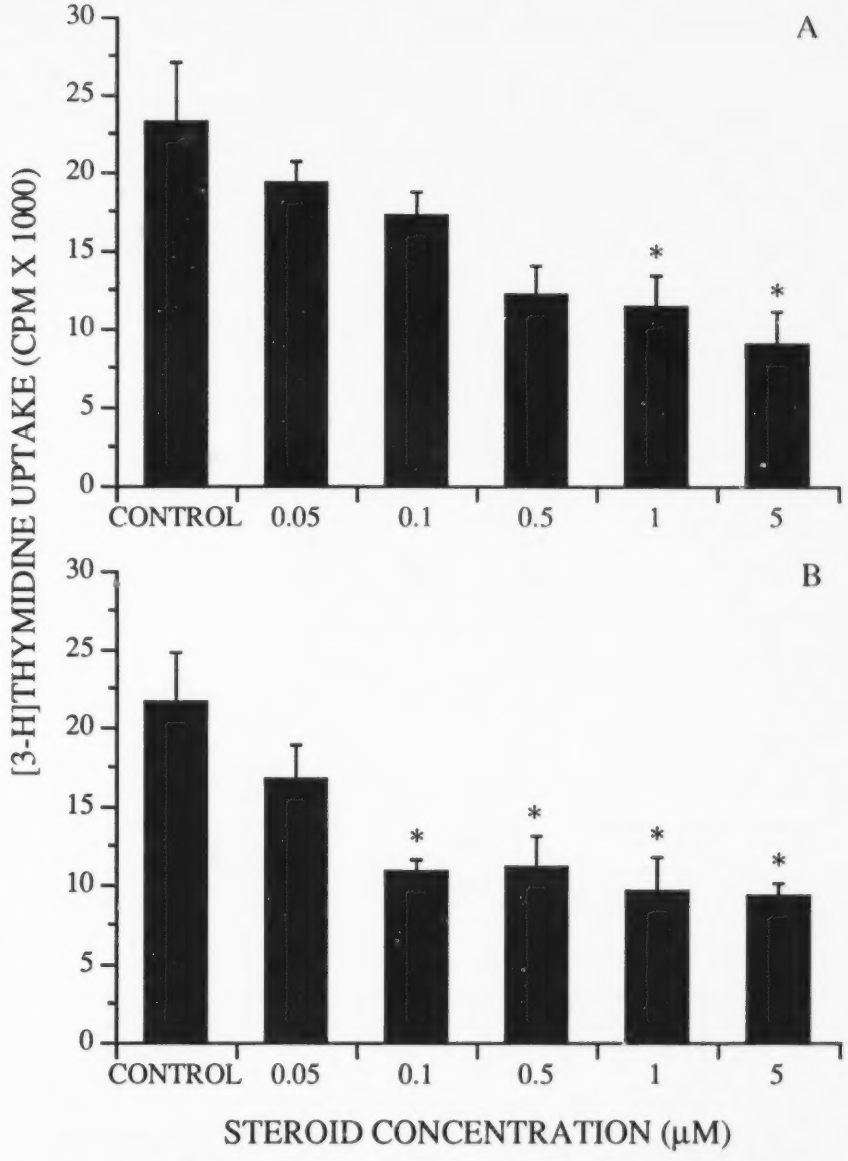

Figure 5-1. The effects of estradiol (A) and cortisol (B) on the mitogen-induced proliferation of goldfish PBL. Each column represents the mean CPM of [^{3}H]thymidine incorporation of the lymphocytes ± SEM of four fish. Stars indicate statistically significant differences between the steroid treated and untreated control groups ($p < 0.05$; t-test).

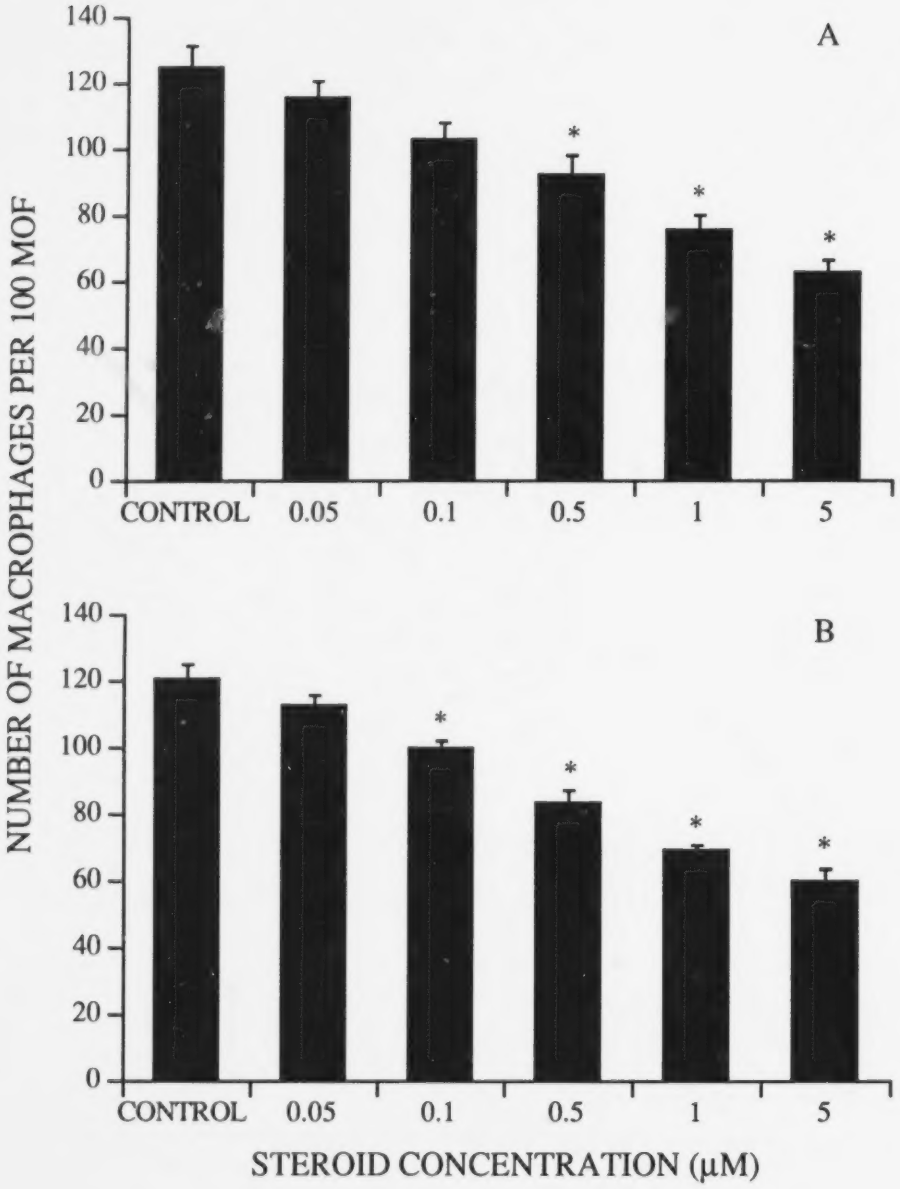

Figure 5-2. Effects of estradiol (A) and cortisol (B) on chemotaxis of cultured goldfish macrophages. Each column represents the mean number of cells that migrated through the pores of the membrane and adhered onto the other side of the membrane ± SEM of a typical experiment (n = 4). The experiment was repeated once. Stars indicate statistically significant differences between the control and steroid treated groups ($p < 0.05$; t-test).

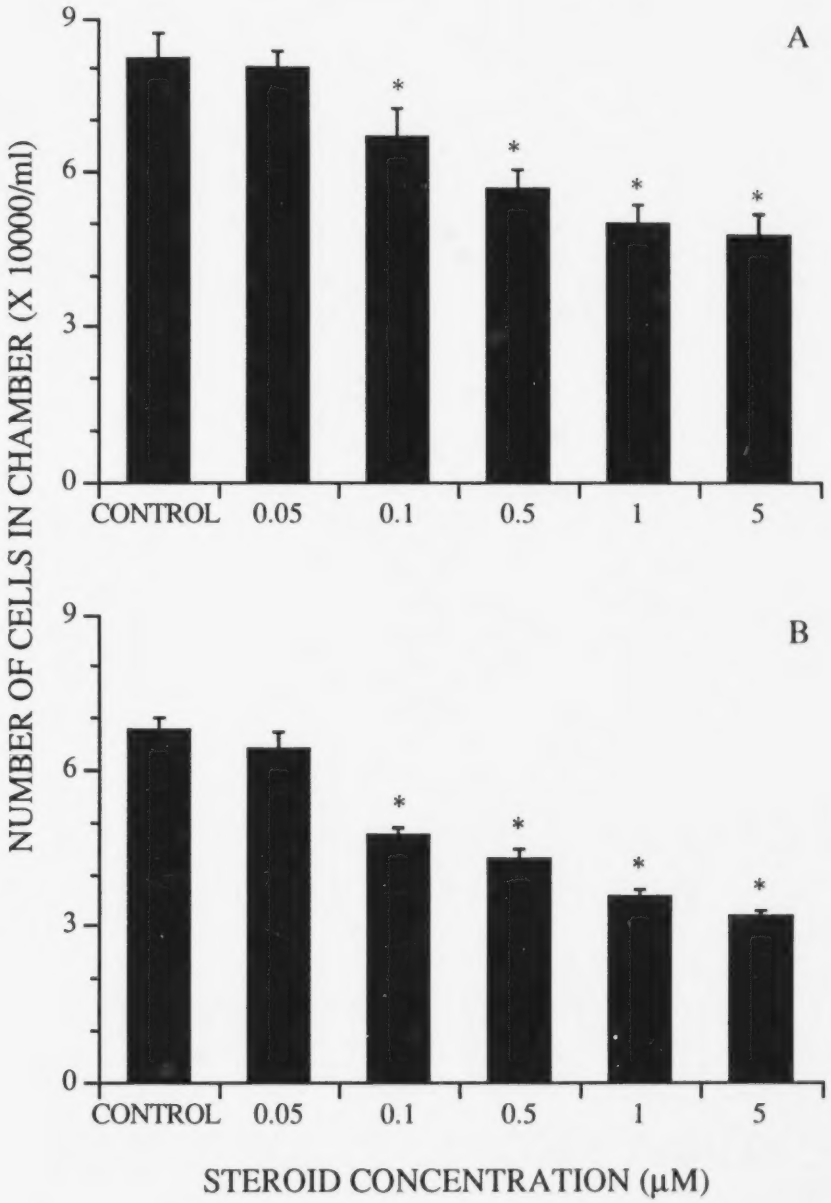

Figure 5-3. Effects of estradiol (A) and cortisol (B) on chemotaxis of cultured goldfish macrophages. Each column represents the mean number of cells that migrated through the pores and into the lower chamber ± SEM of a typical experiment (n = 4). The experiment was repeated once. Stars indicate statistically significant differences between the control and steroid treated groups ($p < 0.05$; t-test).

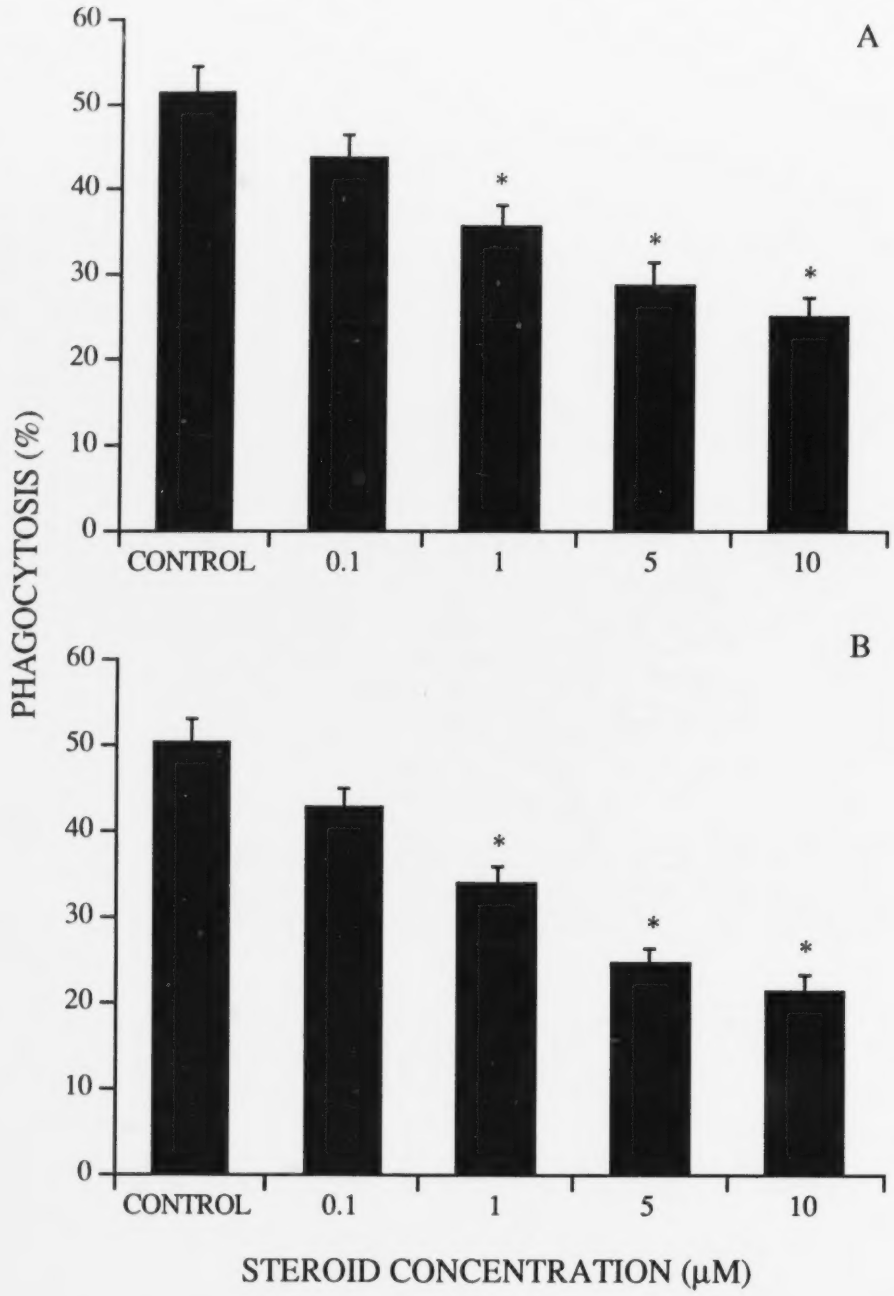

Figure 5-4. Effects of estradiol (A) and cortisol (B) on phagocytosis of opsonized SRBC by goldfish macrophages. Each column represents the mean percent of macrophages with engulfed SRBC ± SEM of a representative of two experiments (n = 3). Stars indicate significant differences between the control and different concentrations of the steroids ($p < 0.05$; t-test).

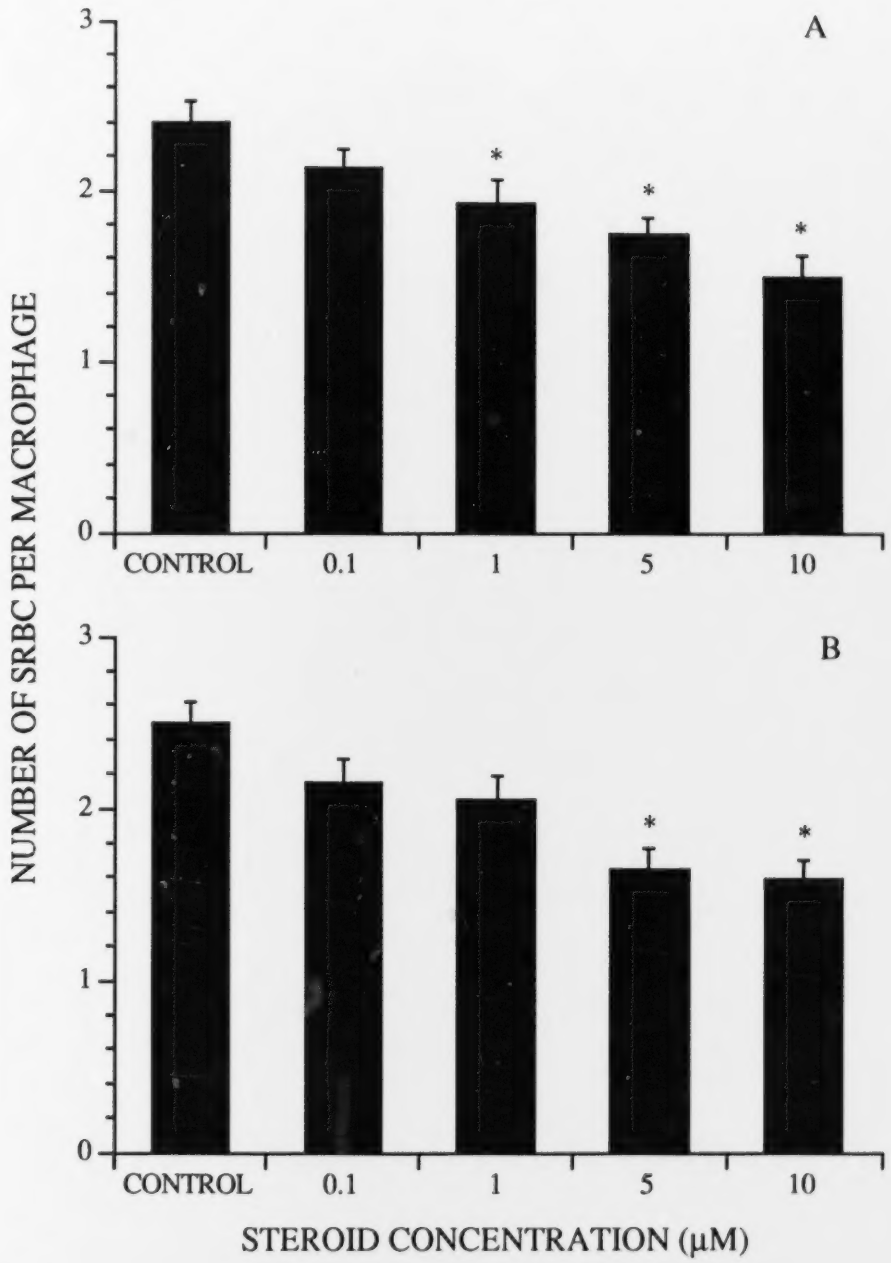

Figure 5-5. Effects of estradiol (A) and cortisol (B) on phagocytosis of opsonized SRBC by goldfish macrophages. Each bar represents the mean number of SRBC in each macrophage ± SEM of a representative of two experiments that showed similar results (n = 3). Stars indicate significant differences between control and estradiol or cortisol treated groups ($p < 0.05$; t-test).

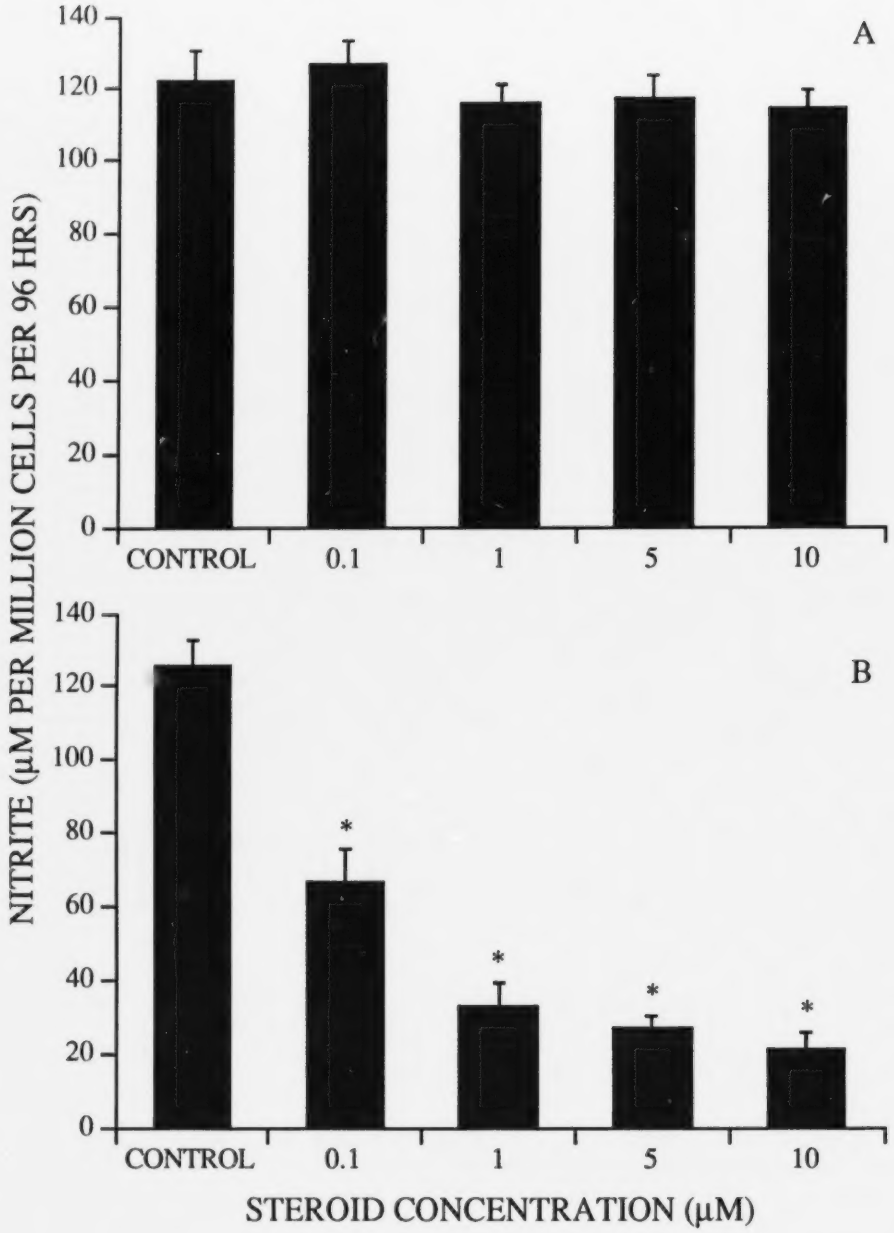

Figure 5-6: Effects of estradiol (A) and cortisol (B) on the LPS stimulated nitric oxide production of goldfish macrophages. Each bar represents the mean µM of nitrite per 10^6 cells per 96 hrs ± SEM of a representative of three experiments that were done in quadruplicates (n = 4). Stars indicate statistically significant differences between control and different concentration of the steroids ($p < 0.05$, t-test).

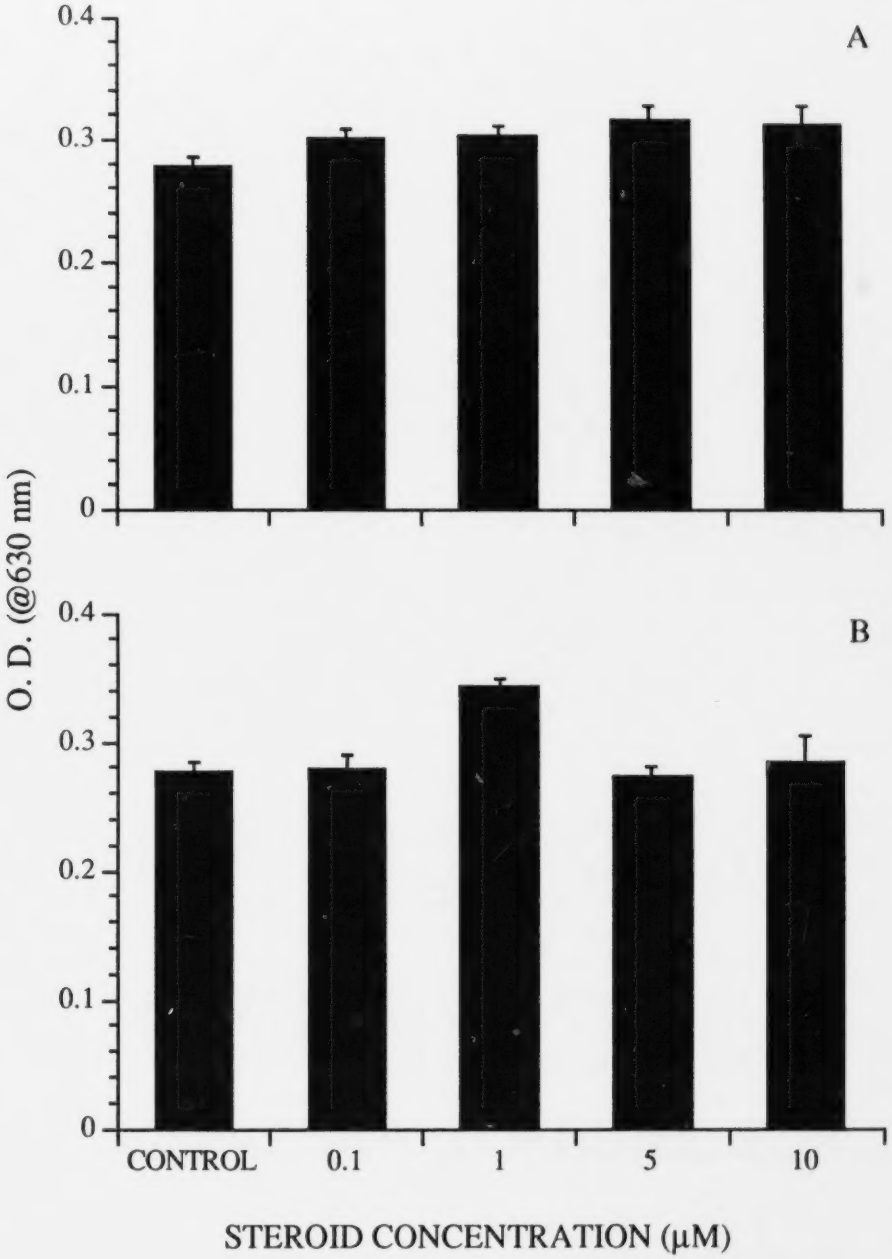

Figure 5-7: Effects of estradiol (A) and cortisol (B) on the mitogen induced O_2^- production of goldfish macrophages. Each bar represents the mean O. D. ± SEM of a triplicate experiment (n = 3). The data are from a single experiment.

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Chapter Six: The Effects of Estradiol Administration on Host Defense against *Trypanosoma danilewskyi* Infection

INTRODUCTION

Accumulating evidence indicates that there are interactions between reproduction and immunity [Grossman, 1984]. The hypothesis that gonadal steroids regulate immune responses has been confirmed by both clinical and experimental observations: 1) there is a sex difference in the immune response against infectious agents and in autoimmune diseases [Carlsten *et al.*, 1992; Schuurs and Verheul, 1990]; 2) gonadectomy and sex steroid replacement alter the immune response [Grossman, 1985]; 3) the immune response is changed during pregnancy at the time when sex steroids are high [Brook-Kaiser *et al.*, 1992; Okamura *et al.*, 1984; Stahn *et al.*, 1978]; 4) leukocytes possess receptors for sex steroids [Jakob *et al.*, 1992; Tabibzadeh and Satyaswaroop, 1989; Weusten *et al.*, 1986].

Sex differences in immune responses have been observed in autoimmune and infectious diseases. For example, lupus erythematosus is more prevalent in females than in males and the process of the disorder is suppressed by androgens and accelerated by estrogens [Grossman, 1985]. In parasitic infection, sex differences have been observed in mice infected with *Leishmania major* [Mock and Nacy, 1988], *Giardia muris* [Daniels, 1993] and in humans infected with *G. lamblia* [Jokipii, 1971]. Estradiol administration significantly enhances the susceptibility and mortality of mice to disseminated gonococcal infection [Kita *et al.*, 1985]. Administration of estradiol inhibits the ability of naive mice to develop immunity against *Plasmodium chabaudi* [Benten *et al.*, 1992], resulting in increases in both the parasitaemia and mortality. However, in mice that are immune to *P. chabaudi*, neither survival rate nor parasitaemia are affected by estradiol, indicating that estradiol suppresses the ability of hosts to acquire immunity but has no effects on the protective immunity [Benten *et al.*, 1992].

Estradiol administration influences the immune system in a number of animal species. A single injection of estradiol decreases thymus weight and causes a transient reduction in circulating thymosin $\alpha 1$ levels in mice [Allen *et al.*, 1984]. In addition, a single injection of estradiol into gilts induces suppression of mitogen-stimulated blood mononuclear cell proliferation [Magnusson and Fossum, 1992]. Estradiol treatment significantly suppresses the bone marrow production of leukocytes and affects the distribution of polymorphonuclear cells in peripheral

blood of mice [Josefsson *et al.*, 1992]. It is postulated that estradiol administration influences the subsequent leukocyte function since estradiol and cortisol have been shown to inhibit the mitogen-induced goldfish lymphocyte proliferation *in vitro* (see Chapter 6).

The concentration of estradiol in goldfish blood is maintained at a barely detectable level during most of the year. The concentration increases to approximately 4 to 6 ng per ml of blood shortly before and during spawning [Kobayashi *et al.*, 1987]. High concentrations of estradiol are toxic to goldfish; when administered at high doses, estradiol causes vitellogenesis in the liver cells in goldfish resulting in high concentrations of vitellin in the blood and a high mortality rate [Kobayashi *et al.*, 1989].

In the present study, the effects of estradiol administration on the course of the primary infection, on the resistance of goldfish to reinfection, and on the mitogen-induced lymphocyte proliferation were examined. Implantation using steroid-filled Silastic implants is a good procedure for administration of estradiol to goldfish. A low, constant concentration of steroids is obtained by using this procedure [Kobayashi *et al.*, 1988]. The estradiol-implanted naive and surviving goldfish were infected with *T. danilewskyi* and the mortality and parasitaemia were examined using the procedure described in Chapter 4. The mitogen-induced proliferation of peripheral blood lymphocytes of the estradiol-implanted goldfish was determined using the procedure described in Chapter 3.

MATERIALS AND METHODS

Fish and Parasites:

Goldfish were purchased from either Ozark Fisheries Inc. or Grassy Forks Fisheries and maintained under the same conditions as described in Chapter 3. The strain of *T. danilewskyi* used in this Chapter was obtained from Dr. Woo and maintained using the same procedure as that described in Chapter 4.

Isolation and Purification of *T. danilewskyi* from Goldfish Blood:

The parasites were isolated from goldfish peripheral blood using Ficoll-Paque gradient centrifugation and purified using the incubation procedures described in Chapter 4.

Hormone Administration:

The methods of Kobayashi and Stacey [1990] were used to administer sex steroids to goldfish. The Silastic™ Tubing (Cat. No. 602-305, Dow Corning Corp., Midland, Michigan) was cut into 3.0 cm sections that were defined as implants. One end of the implant was cut at a 60 degree angle, which made it easier to insert the implant into the peritoneal cavity through a small opening, and sealed with Silastic Adhesive (Silicone Type A, Cat. No. 891; Dow Corning Corp., Midland, Michigan). The implant was filled with crystal estradiol (about 25 mg per implant) and the other end was sealed using the Silastic adhesive. The estradiol-filled implant was washed with 95% ethanol and sterile PBS to remove traces of estradiol on the surface of the implant. Goldfish were anesthetized with 0.05% MS222. The implant was inserted into the peritoneal cavity through a small cut on the abdominal wall. The hormone-implanted goldfish were held in the aquaria for 5 days to allow wound healing. The steroid concentration in goldfish serum was maintained by controlling the length of the implant and the number of implants inserted into individual goldfish. By implanting one 3 cm-long implant, the concentration of estradiol in goldfish serum can be maintained at approximately 2 ng per ml for up to 2 months [Kobayashi and Stacey, 1990].

Infection of Goldfish and Determination of Parasitaemia:

Goldfish (30 to 40 g) were anesthetized using 0.05% MS222 and implanted with estradiol-filled Silastic implant containing 25 mg estradiol. Each fish was inoculated intraperitoneally with 2.5×10^5 trypanosomes in 0.2 ml TDM using a 1 ml syringe fitted with a 25 gauge needle. Infected goldfish were kept in 80L aquaria with flow-through dechlorinated water. Blood samples were collected from the caudal vein of infected fish every 5 days for the assessment of the course of infection. The numbers of parasites in the blood were determined every 5 days by counting four aliquots of diluted blood using a haemocytometer. The parasitaemia was expressed as the Log_{10} number of parasites per ml of blood.

The Estradiol Administration and Challenge Infection:

To determine the influence of estradiol on the parasitaemia and mortality of goldfish upon challenge infection, goldfish surviving primary infection (6 months after the elimination of the primary infection) were implanted intraperitoneally

with estradiol-filled implant. Five days later, the estradiol-implanted fish were inoculated intraperitoneally with 0.2 ml medium containing 2.5 x 10^5 trypanosomes per fish. The parasite numbers in the blood were examined every 10 days until day 30 using the method described above. The mortality of infected fish was checked every 2 days.

Effects of Estradiol Administration on Mitogen-Induced Goldfish Lymphocyte Proliferation:

To assess the effects of estradiol administration on lymphocyte function, goldfish (40 to 50 g body weight) were anesthetized with 0.05% MS222 and implanted intraperitoneally with estradiol-filled implant containing 25 mg estradiol. Ten days later, the fish were anesthetized with MS222 and 1.5 ml of blood was taken from the caudal vein of each fish. The lymphocytes in the blood were isolated using Ficoll-Paque gradient centrifugation. The mitogen-induced proliferation of the cells was determined using [^{3}H]thymidine incorporation assay developed by Caspi *et al.* [1984] and Clem *et al.* [1984].

Experimental Design:

A. Hormone Administration and Parasitaemia:

A total of 36 goldfish (mixed sex) were divided into 3 groups (Group A, B, and C). Each group consisted of 12 fish. Group A fish were implanted with empty Silastic implants serving as a control. Group B fish were implanted with one estradiol-filled Silastic implant and Group C fish were implanted with two steroid-filled Silastic implants. According to the experimental results reported by Kobayashi *et al.* [1989] and Kobayashi and Stacey [1990], the estradiol concentrations in goldfish serum is maintained at 2 ng per ml after implantation of one implant and 4 ng per ml after implantation of 2 implants.

Six fish per group were inoculated intraperitoneally with 2.5 x 10^5 parasites in 200 µl of TDM using a 1 ml syringe. Six fish were not inoculated and served as controls. The number of parasites in the blood of infected fish were determined every 5 days until day 40 using a haemocytometer. The parasitaemia was expressed as the geometric mean number of trypanosomes per ml of blood.

B. Hormone Implantation and Challenge Infection of Surviving Goldfish:

A total of 36 surviving goldfish (12 months after the elimination of the initial infection) were divided into 3 groups designated as Group A, B, and C. Group A fish were implanted with empty Silastic implants serving as a control. Group B fish were implanted with one silastic implant and Group C fish were implanted with two silastic implants. In each group, six fish were inoculated intraperitoneally with 2.5×10^5 parasites in 200 µl of TDM and the other six fish were not inoculated and served as controls. The number of parasites in each infected fish was determined using a haemocytometer every 5 days until day 40 after infection.

C. Estradiol Implantation and Mitogen-Induced Lymphocyte Proliferation:

Eighteen goldfish (40 to 50 g) were divided into 3 groups designated as Group A, B, and C. Group A fish were intraperitoneally implanted with empty implants (control). Group B and Group C fish received 1 and 2 Silastic implants containing estradiol, respectively. After 10 days, peripheral blood was collected from individual fish. Peripheral blood leukocytes were isolated using Ficoll-Paque centrifugation and the mitogen-induced lymphocyte proliferation determined using the assay described in Chapter 3.

RESULTS

Effects of Estradiol Administration on the Parasitaemia of Goldfish Infected with *T. danilewskyi*:

Estradiol implantation significantly increased the parasite numbers in the blood of *T. danilewskyi*-infected goldfish. The average number of parasites in the blood of goldfish with 2 estradiol-containing Silastic implant implants was significantly higher than that of untreated control on days 5, 10, and 15 ($p = 0.048$, $p = 0.05$, $p = 0.049$, respectively; t-test) but not on day 20 after infection (Fig. 6-1). The implantation of only 1 estradiol-filled implant, which maintained approximately 2 ng per ml of estradiol in goldfish blood [Kobayashi *et al.*, 1989; Kobayashi and Stacey, 1990], also resulted in increased number of parasites in the

blood. The average number of parasites in the blood of estradiol implanted fish were consistently higher than those of control fish. Fish with 2 estradiol-filled implants (maintaining approximately 4 to 6 ng estradiol per ml of blood), exhibited higher parasitaemia and mortality when compared to fish with 1 estradiol-filled implant or sham operated controls.

In addition to the higher number of parasites per ml of blood, the parasites appeared earlier in the blood of estradiol treated fish. All fish that received hormone treatment were positive for *T. danilewskyi* on day 5, whereas, only 50% of sham operated control fish had parasites in their blood on day 5 after infection.

The Effects of Estradiol Administration on the Mortality of Goldfish Infected with *T. danilewskyi*:

Estradiol administration not only increased the parasitaemia in treated fish, but also induced higher mortality in animals infected with *T. danilewskyi* (Table 6-1). To examine the mortality of estradiol-implanted goldfish, the hormone-treated fish were infected intraperitoneally with 2.5×10^5 trypanosomes per fish, a dose that usually induces 10% mortality in untreated goldfish. In estradiol-implanted fish, this dose of parasites induced much higher mortality when compared to the sham operated control. Three out of 12 fish (25%) in 1 implant group, and 8 out of 12 fish (67%) in 2 implant group died. Statistical analysis using Chi-square test indicated that there were significant differences between the control and 2 implant groups ($p < 0.0001$) and between 1 implant and 2 implant groups ($p = 0.0064$). These results indicate that estradiol administration significantly increased the mortality of goldfish infected with *T. danilewskyi*.

Effects of Estradiol Administration on Resistance of Surviving Goldfish:

To determine the effects of estradiol on the resistance of goldfish to re-infection, surviving goldfish were implanted intraperitoneally with estradiol-filled implant and challenged with 2.5×10^5 *T. danilewskyi* per fish. Neither the parasitaemias nor the mortality of challenged goldfish was influenced by estradiol administration.

Effects of Estradiol Administration on the Mitogen-induced Proliferation of Goldfish Lymphocytes:

Estradiol was administered to goldfish by intraperitoneal implantation of steroid-containing Silastic implants. Ten days after implantation, the proliferative responses of the peripheral blood leukocytes were examined using the [^{3}H]thymidine incorporation assay. The results showed that estradiol administration significantly inhibited the mitogen-induced proliferative responses of goldfish leukocytes (Fig. 6-2). The mean net value for control group was 15,500 ± 2,000 CPM. A one third decrease was observed in 1 implant group. The mean net value of CPM decreased by two thirds in 2 implant group. Statistical analysis (t-test) showed that there were significant differences between control and 1 implant group ($p = 0.015$), control and 2 implant group ($p = 0.002$), and between 1 and 2 implant groups ($p = 0.017$).

DISCUSSION

Sex differences in autoimmune diseases and in immune responses against infectious diseases have been observed in many vertebrates [Grosmann, 1985]. Certain autoimmune disorders such as idiopathic thrombocytopenic purpura, lupus erythematosus, and rheumatoid arthritis are more prevalent in females than in males. The process of these diseases is depressed by androgens and accelerated by estrogens indicating that sex steroids play an important role in these disorders [Grossman, 1985]. Experimental animals show apparent sex difference in susceptibility to parasitic protozoan infections including *Toxoplasma gondii* [Kittas and Henry, 1980], *P. chabaudi* [Benten *et al.*, 1992], *L. major* [Mock and Nacy, 1988], and *G. muris* [Daniels, 1993].

Estradiol has been demonstrated to modulate immune responses by influencing the functions of mononuclear cells [Benten *et al.*, 1992; Feinberg, 1992; Magnusson and Fossum, 1992] and polymorphonuclear leukocytes [Buyon *et al.*, 1984; Josefsson *et al.*, 1992; Neumann *et al.*, 1987]. All these results were obtained from experiments in mammals. Little is known about the effects of sex steroids on immune responses in fish. Only testosterone has been demonstrated to inhibit specific antibody-producing cells in chinook salmon, *Oncorhynchus*

tshawytscha, as detected by the plaque-forming cell assay [Slater and Schreck, 1993]. The present study established that estradiol can modulate immune responses against parasitic infections in fish.

Estradiol was administered by using estradiol-filled Silastic implants instead of injection used in mammals because a high concentration of estradiol has been demonstrated to be toxic to goldfish [Kobayashi *et al.*, 1987]. For example, administration of estradiol by injection produces a high but transient hormone concentration in goldfish blood, which induces a significant increase of vitellin in the blood, which may be responsible for the high mortality [Pankhurst *et al.*, 1986]. Implantation of hormone-filled implants in the peritoneal cavity maintains a relatively low (2 to 4 ng per ml) but constant (lasting more than 6 weeks) concentration of estradiol in the blood of goldfish [Pankhurst *et al.*, 1986]. The concentration of estradiol in goldfish blood produced by implantation was similar to that of estradiol maintained in the blood of goldfish shortly before and during spawning, which is desirable for our experiments designed to examine the immunomodulatory effects of the hormone at physiological concentrations.

I found that administration of physiological doses of estradiol significantly increased both the parasitaemia and mortality of goldfish infected with *T. danilewskyi*. These results suggest that estradiol increased the susceptibility of goldfish to *T. danilewskyi* infection. The change in susceptibility of goldfish to *T. danilewskyi* after hormone treatment was probably due to immunosuppression. The immuno-suppressive effects of estradiol were confirmed by the *in vitro* findings that estradiol had inhibitory effects on the function of goldfish lymphocytes and macrophages (Chapter 6). The data also support the hypothesis that the higher mortality of fish during spawning might be a result of the increased sex steroids in the blood.

Goldfish that survived the initial infection were implanted with estradiol-filled implants and inoculated intraperitoneally with the parasite. The results showed that estradiol administration neither caused significant rise in parasitaemia nor reduced the survival rate of goldfish infected with *T. danilewskyi* indicating that estradiol did not suppress the existing immunity to the parasite. These results support the findings that estradiol suppresses acquiring, but not protective immunity against *P. chabaudi* infection in mice [Benten *et al.*, 1992]. It has been proposed that the immunosuppressive action of estradiol is a specific genomic effect, that is to say, estradiol-induced gene products prevent the development of or suppress the protective immunity against parasitic infection in mice [Benten *et*

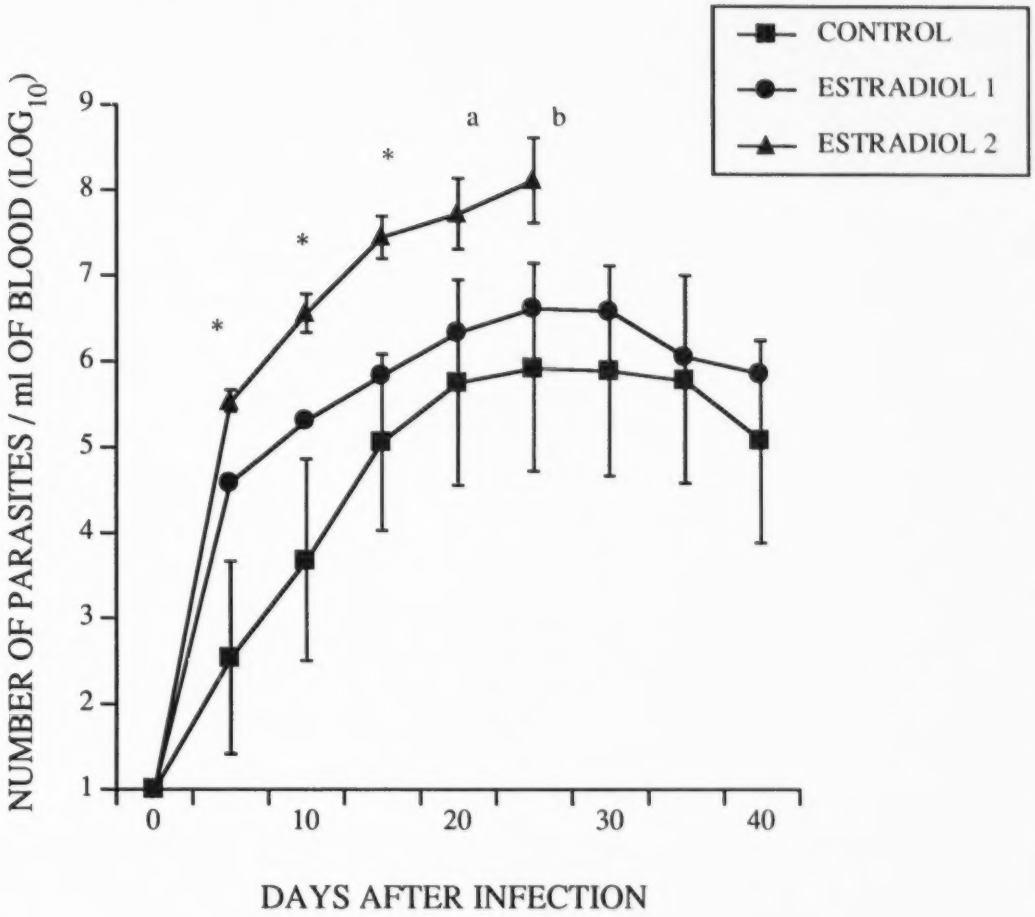
al., 1992]. It is possible that similar mechanisms are operational in the *T. danilewskyi*-goldfish system. Further studies should be conducted to determine which gene products are responsible for the immunosuppressive action of estradiol in *T. danilewskyi* infection.

Estradiol implantation also inhibited the mitogen-induced proliferation of goldfish lymphocytes as determined by [^{3}H]thymidine incorporation. Significant decrease in proliferative responses of the lymphocytes was observed in fish received 1 implant. Further decrease in [^{3}H]thymidine incorporation was seen in fish received 2 implants (Fig. 6-2). These results are in agreement with the previous data showing that estradiol implantation increases the parasitaemia and mortality in goldfish infected with *T. danilewskyi*. These data are also in agreement with the reports that estradiol administration inhibits the *in vitro* mitogen-induced leukocyte proliferation in gilts [Magnusson and Fossum, 1992]. My findings indicate that the immunosuppressive effects of estradiol in goldfish may be mediated, at least in part, by inhibition of lymphocyte function.

In the present study, estradiol implantation has been demonstrated to increase the parasitaemia and mortality in goldfish infected with *T. danilewskyi* and to inhibit the proliferation of goldfish PBL as determined by an *in vitro* [^{3}H]thymidine incorporation assay. These results suggest that estradiol at physiological concentration may have immunosuppressive action in fish, and estradiol may contribute to the higher susceptibility of fish to infectious diseases during spawning.

Table 6-1. The mortality at day 40 of estradiol-treated goldfish infected with *T. danilewskyi*. Two groups of 12 fish were given intraperitoneally one estradiol and two estradiol implants (25 mg/implant), respectively. They were inoculated with 2.5 x 10^5 trypanosomes per fish 5 days after the hormone administration. Two types controls (12 fish per group) were used. Control 1 fish were intraperitoneally implanted with two estradiol implants without infection of the parasites. Control 2 fish were intraperitoneally implanted with two empty implants and inoculated with the parasites. The mortality of fish was recorded every 2 days until day 40 after infection. Statistical analysis using Fisher's exact test indicated significant differences between control 1 and two estradiol implant groups ($p = 0.001$), control 2 and two estradiol implant groups ($p < 0.01$). There were no significant differences between the two control groups ($p = 1.00$), control and one estradiol implant groups ($p = 0.22$), or between one and two estradiol implant groups ($p = 0.10$).

TREATMENT	MORTALITY	
	# Dead/# Inoculated	(%)
CONTROL 1	0/12	0
CONTROL 2	1/12	8.3
1 ESTR. IMPLANT	3/12	25.0
2 ESTR. IMPLANTS	8/12	66.7



a One out of 6 fish died on day 17.
b Three out of 6 fish died on day 25.

Figure 6-1. Influence of estradiol on the parasitaemia of goldfish infected with 2.5 x 10^5 *T. danilewskyi* per fish. Each point represents the geometric mean number of parasites per milliliter of blood ± SEM of 6 goldfish (triplicate counts for each fish). Stars indicate statistically significant differences between fish with 2 estradiol containing implants and fish with control implants ($p < 0.05$; t-test). For clarity, error bars for Estradiol 1 group are not shown.

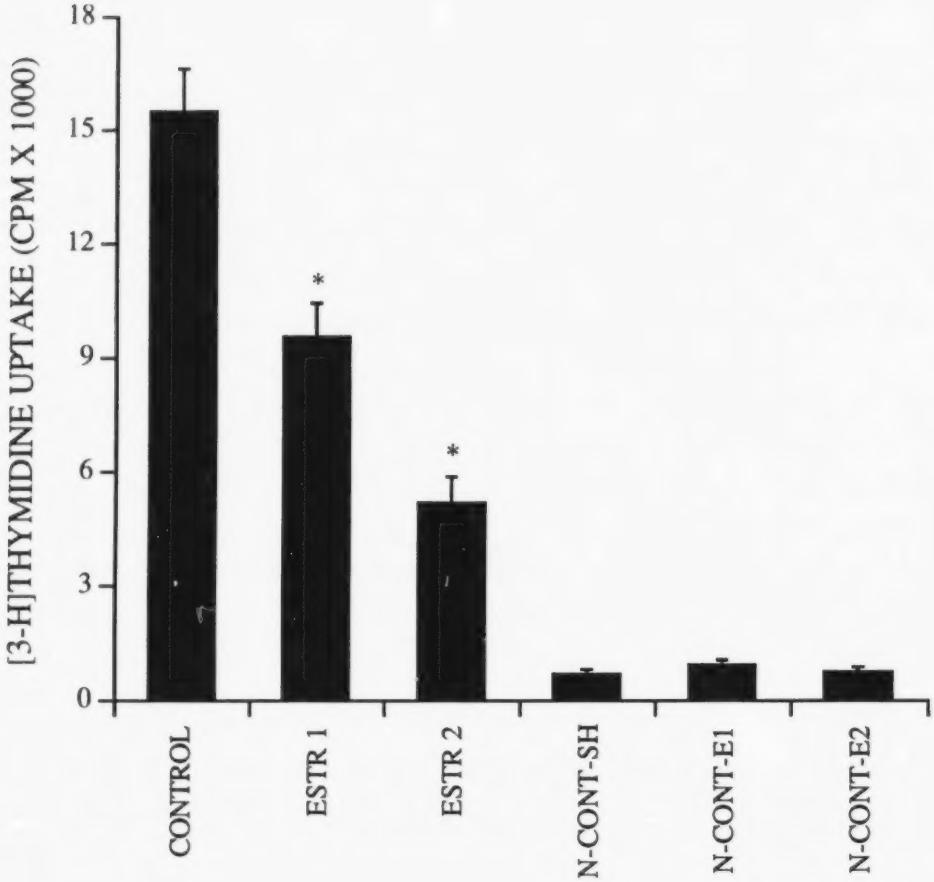


Figure 6-2. The effects of estradiol on the mitogen-induced lymphocyte proliferation of goldfish. The fish in control group received empty implants. Estradiol 1 group fish received 1 estradiol-containing implant. Estradiol 2 group fish received 2 estradiol-containing implants. N-CONT-SH, N-CONT-E1 and N-CONT-E2 groups are the no mitogen controls for the sham operated control, estradiol 1 and estradiol 2 groups, respectively. Each column represents the mean CPM ± SEM in [^{3}H]thymidine incorporation into lymphocytes of 6 fish. Stars indicate the significant differences between control and estradiol-treated groups ($p < 0.05$; t-test).

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Chapter Seven: General Discussion

Sex differences in immune responses against infectious diseases including parasitic infections and in autoimmune diseases have been well documented in mammals [Benten *et al.*, 1992; Grosmann, 1984; 1985; Magnusson and Fossum, 1992]. Based on the information obtained in the studies of steroid-immune interactions in mammals, I hypothesized that sex steroids may have immunoregulatory action in fish and sex hormone administration would increase the susceptibility of fish to various pathogens. To verify this hypothesis, experiments were conducted to answer two main questions: (1) Do estradiol and cortisol inhibit the function of goldfish lymphocytes and macrophages *in vitro*? and (2) Does estradiol administration increase the susceptibility of goldfish to *T. danilewskyi* infection? To answer these questions, the immunological assays for determination of the normal function of goldfish lymphocytes and macrophages *in vitro* were developed and the course of *T. danilewskyi* infection in goldfish characterized.

SYNOPSIS OF THE RESULTS

Lymphocytes and macrophages are two major players in specific immune responses against infectious diseases in fish. Consequently, I assessed the effects of estradiol on the function of goldfish lymphocytes and macrophages in an attempt to define the immunoregulatory properties of estradiol in fish. For this purpose, I adapted a series of immunoassays including mitogen-induced proliferation, chemotaxis, phagocytosis, O_2^- and nitric oxide production for goldfish lymphocytes and macrophages.

(1) Steroids and Goldfish Lymphocyte Proliferation: A mitogen-induced proliferation assay for goldfish lymphocytes was developed. This assay uses [^{3}H]thymidine incorporation to measure the synthesis of DNA by lymphocytes in the process of mitosis. The proliferative response is an important property of lymphocytes since it is the cellular basis for the development of specific and non-specific immunity. Goldfish PBL exhibited a strong proliferative response when stimulated with PMA and A23187. However, unlike PBL of channel catfish [Miller and Clem, 1988], goldfish PBL showed low but significant proliferation in response to PMA indicating species differences in mitogen responses in fish.

Estradiol and cortisol significantly inhibited the proliferation of goldfish lymphocytes *in vitro*. Estradiol administration also inhibited the proliferation of lymphocytes as determined using an *in vitro* [^{3}H]thymidine incorporation assay. These results suggested that estradiol and cortisol may exert their immunoregulatory action, at least in part, by inhibiting the proliferation of lymphocytes.

(2) Steroids and Goldfish Macrophage Functions: I established a goldfish macrophage cell line and developed several macrophage assays: chemotaxis, phagocytosis, nitric oxide and O_2^- production. The findings indicated that goldfish macrophages exhibit oriented migration towards a chemoattractant, actively engulf target cells, and synthesize and release reactive oxygen and nitrogen intermediates when appropriately stimulated.

The influence of estradiol and cortisol on macrophage function differed. Both estradiol and cortisol inhibited chemotaxis and phagocytosis of the cells and had no effects on O_2^- production. However, cortisol, but not estradiol, strongly inhibited nitric oxide production of the cells. These results suggest that estradiol and cortisol may suppress non-specific and specific immune, as well as inflammatory responses by inhibiting the function of macrophages. It appears that the mechanisms of their action may be different.

(3) Steroids and *T. danilewskyi* Infection in Goldfish: Goldfish showed a dose-dependent response to *T. danilewskyi* infection. Higher doses caused higher mortality. The dose-dependent mortality observed in the present study is in agreement with the data reported by Woo [1981]. Estradiol administration significantly increased the parasitaemia and mortality of goldfish in the primary infection with *T. danilewskyi*. However, estradiol implantation failed to influence the parasitaemia and mortality of surviving fish after a challenge infection. These findings confirm previous *in vitro* results that estradiol suppresses the function of lymphocytes and macrophages in goldfish, and are in agreement with the data reported by Benten and colleagues [1992] who demonstrated that estradiol injection increases the parasitaemia and mortality of mice in the primary but not in the challenge infection with *P. chabaudi*.

ESTRADIOL ACTION ON TELEOST IMMUNE RESPONSES

The data in this thesis revealed that estradiol inhibited lymphocyte proliferation and macrophage function and induced higher parasitaemia and mortality in

goldfish infected with *T. danilewskyi*. These results are not unanticipated because several lines of evidence indicate that sex steroids have miscellaneous effects on the immune system of mammals. For example, hexoestrol administration induces severe thymic atrophy and increases the mortality in gonadectomized mice infected with *T. gondii* [Kittas and Henry, 1980]. Estradiol administration significantly suppresses the inflammatory response as measured by footpad swelling induced by cholera toxin and documented by histologic examination. The mechanisms whereby estrogen exerts its anti-inflammatory property are mediated by directly inhibiting leukocyte production in bone marrow and by affecting the distribution of polymorphonuclear cells in peripheral blood [Josefsson *et al.*, 1992]. Estradiol has also been demonstrated to decrease the expression of MHC antigens on guinea pig blood mononuclear leukocytes [Debout *et al.*, 1991]. Estradiol injection significantly increases bacteremia and mortality of mice in gonococcal infection by inhibition of bactericidal activity of polymorphonuclear leukocytes responsible for the elimination of the bacteria [Kita *et al.*, 1985]. Estradiol at prepartum concentrations (3000 pmol/l) significantly inhibits phagocytosis of porcine polymorphonuclear leukocytes [Magnusson, 1991]. These studies provide strong evidence for an immunosuppressive role for estradiol in mammals. The results of the present study indicate that sex steroids have immunoregulatory properties in host defense of goldfish against parasitic protozoa.

Estradiol in goldfish blood is maintained at a barely detectable level to 1 ng per ml of blood during most of the year and drastically increases to 4-6 ng per ml of blood shortly before and during spawning [Kobayashi *et al.*, 1988]. The results of the present study suggested that the increase in estradiol in goldfish blood may contribute to the higher susceptibility of goldfish to infectious diseases during spawning. Testosterone in goldfish blood also increases along with estradiol during spawning [Kobayashi *et al.*, 1987] and the immunosuppressive action of testosterone in fish has been established [Slater and Schreck, 1993]. It is possible that testosterone inhibits the immune response in goldfish, and these two steroids may act synergistically in inhibition of immune responses in goldfish. In addition, spawning is stressful resulting in high concentration of glucocorticoids in the blood of the animal [Pickering, 1981]. Thus, glucocorticoids may also play a role in inhibition of immune responses in goldfish.

CORTISOL ACTION ON TELEOST IMMUNE RESPONSES

The immunosuppressive action of glucocorticoids has been well defined in most vertebrates including teleosts. Thus, it is hardly surprising that cortisol exhibited suppressive effects on lymphocyte proliferation and macrophage functions in goldfish. A large body of literature demonstrates that glucocorticoids, including both physiological (e.g. cortisol) and pharmacological (e.g. dexamethasone) steroids, have immunosuppressive and anti-inflammatory actions in a variety of animals. Glucocorticoids affect a diversity of lymphocyte functions including inhibition of lymphocyte proliferation [Deitch *et al.*, 1987; Magnusson and Fossum, 1992; Rondinone *et al.*, 1992], suppression of lymphokine production [Byron *et al.*, 1992; Rolfe *et al.*, 1992], induction of apoptosis [Cohen, 1992; Garvy *et al.*, 1993; Gruol and Altschmied, 1993; Sun *et al.*, 1992], and alteration of metabolic activities [Osterland *et al.*, 1992; Serrano and Curi, 1992]. Cortisol suppresses human NK cell activity [Holbrook *et al.*, 1983; Gatti *et al.*, 1987]. Glucocorticoids exert their anti-inflammatory effects by modulating the function of granulocytes. For example, dexamethasone inhibits neutrophil chemotaxis [Kurihara *et al.*, 1984] and nitric oxide synthase (NOS) [McCall *et al.*, 1991]. Glucocorticoids inhibit cytokine-induced eosinophil survival [Wallen *et al.*, 1991] and responses to granulocyte-macrophage colony-stimulating factor [Lamas *et al.*, 1991]. Glucocorticoids also inhibit anti-IgE-induced histamine release from human basophils [Bergstrand *et al.*, 1984; Schleimer *et al.*, 1989]. Prednisolone and dexamethasone inhibit IL-1α and IL-1β production by human monocytes [Amano *et al.*, 1993; Lew *et al.*, 1988]. These data indicate that glucocorticoids influence the function of all type of leukocytes in mammals. Receptors for glucocorticoids have been identified in mammalian leukocytes including polymorphonuclear and mononuclear leukocytes [Altman *et al.*, 1981; Salkowski and Vogel, 1992] .

Several lines of evidence indicate that glucocorticoids have immunoregulatory action in fish. Cortisol has been reported to suppress the mitogen-induced lymphocyte proliferation in plaice [Grimm, 1985] and trout [Tripp *et al.*, 1987]. Cortisol injection causes significant reduction of inflammatory cells in the peritoneal cavity of plaice [MacArthur *et al.*, 1985]. Cortisol treatment suppresses the chemiluminescent response of stripped bass phagocytes after stimulation with PMA [Stave and Robertson, 1985]. Receptors for glucocorticoids have been

detected in salmon leukocytes and the affinity of the receptors is lower in stressed or cortisol-treated salmon [Maule and Schreck, 1990; 1991]. Seasonal fluctuation in the affinity and number of the receptors has been observed in coho salmon [Maule *et al.*, 1993]. My data support the evidence that permeates the literature that cortisol has suppressive effect on fish lymphocytes and macrophages.

FUTURE RESEARCH

In chapter 3, I described the establishment and characterization of a goldfish macrophage cell line and the development of immunoassays for goldfish lymphocytes and macrophages. These macrophages can be used as both effector and target cells in immunological and physiological studies. Cytokine production by leukocytes and immunoregulatory properties of cytokines have been well documented in mammals but little is known about their function in fish. The macrophages can be used as targets for detection of cytokine activities in fish leukocyte supernatants. For example, IFN γ-like activity has been demonstrated in trout leukocytes stimulated with Con A and PMA using primary cultures of trout head kidney macrophages as target cells [Graham and Secombes, 1988]. Demonstration of the production of IFN γ or macrophage activating factor by goldfish leukocytes will contribute to cytokine studies in fish. The macrophage cell line will facilitate this study. In addition, the cell line can be used as effector cells to investigate the cytotoxicity, IL-1 and TNF production of fish macrophages.

The antigen presenting function of macrophages has been well defined in mammals but in fish the only explored species is channel catfish [Vallejo *et al.*, 1991; 1992]. Confirmation of the antigen presenting function of goldfish macrophages will provide important information about the immune responses in fish.

The data of this study showed that male goldfish are more susceptible to *T. danilewskyi* infection than females suggesting that male sex hormone testosterone, like estrogens and glucocorticoids, may play a role in regulating host immune response against parasitic infection. Testosterone has suppressive effects on antibody production of lymphocytes in salmon [Slater and Schreck, 1993]. The immunoregulatory properties of testosterone in the *T. danilewskyi*-goldfish model system established in this study should be investigated.

The receptors for glucocorticoids and sex steroids have been identified in mammalian leukocytes. Glucocorticoid receptors have also been demonstrated in

fish leukocytes [Maule and Schreck, 1990]. The data of the present study and in the literature [Slater and Schreck, 1993] indicate that estradiol and testosterone influence leukocyte function in fish. The basis for hormone effect on target cells is the presence of receptors for the hormone on/in the targets. Identification of sex steroid receptors in fish leukocytes will provide a solid basis for the immunoregulatory properties of sex steroids in fish. The macrophage cell line will greatly benefit the study of sex steroid receptors in fish leukocytes.

Production of nitric oxide by mammalian cells is dependent on the presence of L-arginine and is catalyzed by an inducible enzyme known as NOS. This enzyme has been cloned and sequenced in the human, rat and mouse, showing significant differences in the time course of mRNA expression of the enzyme and in response to cytokines and/or LPS between species [Nussler and Billiar, 1993]. The present study showed that the fish macrophage cell line produces nitric oxide after stimulation with bacterial LPS indicating that fish cells, like mammalian cells, possess inducible NOS. The evolutionary aspects of this effector molecule of the immune system can be easily examined using the model developed in this study. Determination of inducible NOS in fish leukocytes will also contribute to our knowledge of fish immune responses against infectious diseases and tumor development and in the future it may provide a better understanding of the macrophage antimicrobial mechanisms. The understanding of these mechanisms will be invaluable in our attempts to control infectious diseases in fish aquaculture.

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APPENDIX

A. Chemicals, Media and Prepared Solutions

Adenosine, Cat. No. A-4036[1]
Ammonium chloride, Cat. No. B27149[2]
Calcium ionophore (A23187), Cat. No. C-7522[1]
Concanavalin A (Con A), Cat. No. C-0412[1]
Cytidine, Cat. No. C-4654[1]
D-glucose, Cat. No. ACS 369[2]
Dimethyl sulphoxide, Cat. No. ACS 306[2]
Disodium EDTA, Cat. No. ED2SS[1]
Dulbecco's Modified Eagle Medium, Cat. No. 430-2100 (without sodium bicarbonate)[3]
Estradiol-17β, Cat. No. E-2758[1]
Estradiol-water soluble, Cat. No. E-4389[1]
Ficoll-paque, Cat. No. 17-0840-02[4]
Gentamicin, Cat. No. 600-5750AS (50 mg/ml)[3]
Glutaraldehyde, Cat. No. G5882[1]
Glycerin, Cat. No. G-33B Fisher[5]
Guanosine, Cat. No. G-6264[1]
HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), Cat. No. 845-1344IM[3]
Hydrocortisone-water soluble, Cat. No. H-0396[1]
Hypoxanthine, Cat. No. H-9636[1]
Insulin, Cat. No. I-6634[1]
L-glutamine solution, 200 mM, Cat. No. 320-5030[3]
Leibovitz's L-15 Medium, Cat. No. 430-1300[3]
Lipopolysaccharide, (LPS from *S. typhimurum*), Cat No. 3125-25[6]
MEM amino acid solution, 50 x, Cat. No. 320-1130[3]
MEM nonessential amino acid solution, 100 x, Cat. No. 320-1140[3]

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[1] SIGMA Chemical Co., St. Louis. MO.
[2] BDH Inc. Edmonton, Alberta, Canada.
[3] GIBCO, Burlinton, Ontario, Canada.
[4] Pharmacia LKB Biotechnology Inc. Piscatway, NJ.
[5] FISHER Scientific Co. Fair Lawn, NJ.
[6] Difco Laboratories, Inc. Detroit, MI.

MEM vitamin solution, 100 x, Cat. No. 320-1120AG[3]
N-(1-naphthyl) ethylendiamine, Cat. No. N-9125[1]
Phenol red, Cat. No. P-5530[1]
Phorbol 12-myristate-13-acetate (PMA), Cat. No. P-8139[1]
Potassium dihydrogen orthophosphate, Cat. No. ACS 657[2]
Potassium hydrogen carbonate, Cat. No. ACS 666[2]
Potassium hydroxide, Cat. No. ACS 678[2]
Sodium bicarbonate, Cat. No. B3[illegible]51[2]
Sodium chloride, Cat. No. ACS 78[illegible]
Sodium dihydrogen orthophosphate, Cat. No. ASC 795[2]
Sodium dodecyl sulfate[7]
Sodium ethylenediaminetetraacetic acid, Cat. No. ED2SS[1]
Sodium hydrogen orthophosphate, Cat. No. ACS 807[2]
Sodium hydroxide, Cat. No. ACS 816[2]
Sodium nitrite, Cat. No. S-2252[1]
Sodium pyruvate solution, 100 mM, Cat. No. 320-1360[3]
Sulfanilamide, Cat. No. S-9251[1]
Thymidine, Cat, No. T-1895[1]
Tricaine methanesulfonate (MS222), Cat. No. 18323[8]
Trypan blue stain, 0.4%, Cat. No. 630-5250AG[3]
Uridine, Cat. No. U-3003[1]

B. Preparation of Solutions and Media

1. Modified 10 x Hank's solution

a. Dissolve in 1000 ml Milli Q® water the following:

KCl	4.0 g
KH_2PO_4	0.6 g
NaCl	8.0 g
Na_2HPO_4	0.48 g
D-Glucose	10.0 g
Phenol red	0.1 g

[7] Bethesda Research Laboratories, Bethesda, MD.
[8] Syndel Laboratories Ltd. Vancouver, B. C. Canada.
® Endotoxin-free, de-ionized water prepared using Millipore ion exchange equipment.

b. Stir to dissolve using a magnetic stirrer (final pH 7.2).
c. Filter to sterilize using 0.22 µm filter and stored at 4°C.

2. Nucleic acid precursor solution (2.5 mM)

a. Dissolve the following reagents in 500 ml Milli Q® water:

Adenosine	0.334 g
Cytidine	0.304 g
Guanosine	0.354 g
Hypoxanthine	0.170 g
Thymidine	0.303 g
Uridine	0.305 g

b. Heat in 56°C water bath to dissolve.
c. Sterilize using 0.22 µm filter and stored at -20°C in 10-ml aliquots.

3. Lysing Buffer

a. In 500 ml of Milli Q® water dissolve the following:

NH_4Cl	4.18 g
$KHCO_3$	0.5 g
Na_2-EDTA	0.02 g

b. Stir to dissolve, filter to sterilize and store at 4°C.

4. GFL-15 Medium

a. 499 ml Leibovitz's L-15 Medium (L-15).
b. 499 ml Dulbecco's Modified Eagle Medium (D-MEM).
c. 1.0 ml gentamicin (50 mg/ml).
d. 1.0 ml 50 mM 2-mercaptoethanol (2-ME, freshly prepared).
e. Adjust to pH 7.4 with 1N NaOH or HCl.
f. Filter to sterilize using 0.22 µm filter and store at 4°C.